STABILIZATION OF SELECTED ORGANIC SUBSTRATES BY SEWAGE LAGOON BACTERIA AS A FUNCTION OF DISSOLVED OXYGEN CONCENTRATION

BY

WILLIAM STEVEN BASHUCKY

A Thesis

submitted to

the Faculty of Graduate Studies and Research

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In partial fulfilment

of the requirements for the degree of

Master of Science

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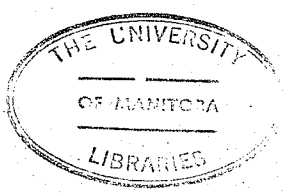

ABSTRACT

The effect of dissolved oxygen (DO) concentration on the stabilization of organic compounds by bacteria indigenous to a mid-continental sewage lagoon was studied. Resting cell suspensions of bacteria recovered from lagoon water for both summer and winter conditions were subjected to automatically controlled dissolved oxygen in the presence of selected substrates. Dissolved oxygen levels that would be non rate-limiting for oxidative breakdown were determined.

For summer samples at 25°C the highest DO concentration required of the substrates tested was $\simeq$ 2 ppm for propionate oxidation. A DO concentration of 0.5 ppm did not limit degradation of butyrate and ethanol. LAS detergent, phenol and acetate utilization was not affected by a DO concentration of 0.24 ppm.

The DO requirement of winter samples at 2°C for propionate was lower, being $> 1.0 \leqslant 1.5$ ppm. A concentration of 0.5 ppm DO did not retard the rate of breakdown of acetate, butyrate, ethanol, phenol and benzoate. LAS was not metabolized by winter bacteria.

When a suspension of psychrotrophic lagoon bacteria was challenged with a mixture of substrates under fully aerobic conditions there was an indication of a diauxic effect.

A manometric procedure to correct for fluctuations in biomass provided a reasonable estimate of bacterial activity. The method was capable of generating an activity constant for the rate of breakdown of test substrates by lagoon bacteria.

The effect of DO concentration upon sewage lagoon management as a function of seasonal change is discussed.

ACKNOWLEDGEMENTS

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INTRODUCTION

INTRODUCTION

Strict aerobic microorganisms require certain minimum or "critical" levels of dissolved oxygen (DO) in the suspending medium in order to carry out various oxygen-dependent metabolic functions. The greater proportion of information in this area has been obtained from studies employing pure cultures. In wastewater treatment processes the resident microflora represents a great diversity of physiological and taxonomic types. Each species will demonstrate a particular oxygen requirement for each oxygen-dependent substrate it is able to metabolize. But it is of great practical importance that the overall oxygen demand of the heterogeneous population be met to achieve efficient wastes purification where a wide variety of substrates is available.

The practice of artifically aerating sewage lagoons to augment the supply of oxygen has increased in recent years. The operation and design of such systems has been controlled mainly by engineering technology with little consideration given to the actual biological requirement for dissolved oxygen. The present study was

undertaken to explore the oxygen requirements of mixed cultures of sewage lagoon bacteria. Experiments were carried out to determine the minimum dissolved oxygen levels that would support maximum rates of breakdown of selected substrates by bacteria indigenous to a sewage lagoon.

HISTORICAL

HISTORICAL

In biological oxidations molecular oxygen acts as a principal terminal electron acceptor in the energy yielding metabolism of substrates by microorganisms. Since dissolved oxygen (DO) concentration is one of the parameters frequently controlled in industrial operations involving microbial activity such as waste treatment processes it is desirable that the oxygen requirements of the microbial agents be known.

Considerable research effort in both basic and applied fields has been expended in studying the effect of DO concentration on microbial growth and metabolism. Early workers (21, 48, 79) using manometric techniques with resting cell suspensions of bacteria and yeast observed that at oxygen concentrations below a threshold value the rate of oxygen uptake by cells decreased. The point at which respiration rate became limited by oxygen concentration was termed the "critical oxygen tension" by Gerard and Falk (30). Wide variations in critical tensions reported were later partly attributed to measurement of partial pressure of oxygen in the gas phase over the culture rather than dissolved oxygen. It

is now known (38) that in systems of this type, resistance to oxygen diffusion at the gas-liquid interface can result in dissolved oxygen tensions in solution being lower than the partial pressure in the gas phase.

Direct measurement of DO was first achieved by Baumberger (2) in 1939. He employed a dropping mercury electrode in studying washed yeast suspensions and concluded that oxygen uptake was independent of oxygen concentration. Winzler (93), using the same technique on yeast cells, found the critical DO concentration in the absence of an energy source to be very low being 0.01 mg/l DO at 30°C (0.2 mmHg)[1], while with substrate present it was ten times higher. He believed the rate-limiting process for respiration at low DO concentrations to be the rate of combination of oxygen with the oxygen transferring enzyme, presumably cytochrome oxidase.

From the determination of apparent K_m (Michaelis constant) values for oxygen in intact cells and cell-free extracts of large bacteria (Bacillus megaterium) and small bacteria (Klebsiella aerogenes), Longmuir (50) concluded that diffusion of oxygen through the cell material to oxidative sites was at least partly rate-

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[1]The concentration of DO may be expressed in mg/l which is ≡ ppm; the corresponding equivalent partial pressure of DO is in mmHg and is referred to as DO tension.

limiting at low oxygen concentrations.

It was later recognized by Johnson (46) that at higher DO levels when uptake is independent of concentration, respiration is controlled by some factor other than cytochrome saturation. This factor, which was termed the "oxygen demand of the cell" is regulated in part by the supply of intermediary metabolites such as adenosine diphosphate (ADP) and reduced nicotinamide adenine dinucleotide ($NADH + H^+$). Johnson also pointed out that the quantity of respiratory enzyme, which is likely to vary with growth conditions, could restrict the ability of the cells to utilize oxygen. He predicted, as had earlier workers (2, 13, 50, 93), that the regulation of metabolism by dissolved oxygen would be in the form of a single enzyme-catalyzed reaction. That is, when the DO falls below the critical level, the lack of saturation of the respiratory enzyme with oxygen results in a lowered reaction velocity so that the rate of reductant supply is not sufficient to meet the oxygen demands of the cell. He maintained that when low levels of oxygen are present, respiration might become limited by the resistance to oxygen diffusion presented by some intracellular barrier. It has been shown (4) that extracellular diffusion of oxygen from the bulk of the

suspending medium to cell surfaces could be regarded as being negligible in most agitated microbial systems.

Recently, Harrison (38) has proposed that data leading to Johnson's hypotheses could equally well be explained by the multi-enzyme model presented by Chance (14) which takes the interactions of the whole respiratory chain into account. In this model, the first component of the chain, the terminal oxidase, starts to become reduced at oxygen concentrations above the critical level and the second and third components also display some reduction. The fourth component, however, remains more or less in a steady state until the critical oxygen level is reached.

Investigation into the adaptive response of growing microorganisms to DO concentration was greatly aided by the application of continuous culture techniques and the development of reliable dissolved oxygen electrodes. This led to work in 1966 by Maclennan and Pirt (53) who outlined a method for automatically controlling the DO concentration in stirred microbial cultures over the range of 0.01 - 1.4 mg/l DO at 30^{o}C (0.26 - 30 mm$Hg0_2$) by changing the partial pressure of oxygen in the gas phase while keeping the total flow constant. It was not until 1964 that the problems of instability and low output currents associated with earlier oxygen

probes (10, 51) were overcome. In that year Mackereth (52) described an improved membrane bound galvanic cell which was selectively permeable to oxygen in solution and which remained stable for long periods of time. An autoclaveable version of the electrode has also been developed (89).

Harrison and Pirt (39) were the first to use such a membrane electrode in studies into the effect of oxygen on the metabolism of microorganisms in continuous culture. They found that for the facultative anaerobe Klebsiella aerogenes NCTB 8017, there was a critical DO between 0.46 - 0.7 mg/l DO at 30°C (10 - 15 mmHg) above which respiration rate was constant. Below this level the organism demonstrated a stimulation of oxygen uptake and the products of glucose metabolism started to change to those typical of anaerobic metabolism. Increased rates of respiration under conditions of low oxygen concentration have been further reported for Klebsiella aerogenes (41, 42, 43), and also for Escherichia coli (41, 42, 61), Pseudomonas AM1 (55) and Hemophilus parainfluenzae (92). The same phenomenon has also been observed under certain conditions in the fungus Aspergillus nidulans (12) and in the yeast Candida utilis (62).

Prompted by their own observations (39) and those of others, Harrison and Pirt modified the original (30) definition of critical DO tension to become: "the oxygen tension above which the respiration rate of an organism is independent of changes in DO; below this tension the oxygen uptake rate of the organism may increase or decrease in response to a decrease in oxygen tension, according to cultural conditions".

The increase in oxygen uptake at low DO has been ascribed to various causes by different authors, with the recognition that the overall mechanism is one of controlling cellular energy balances. Harrison and Pirt (39) theorized that this stimulation might be caused by a decrease in ATP production resulting from either an alternate electron transport pathway becoming operational or a loss of coupling at a site of oxidative phosphorylation. Harrison and Maitra (43) expanded on this and suggested that energy production via oxidative phosphorylation becomes less efficient at low DO concentrations and that an increased rate of respiration is required to maintain a tight control over the ATP/ADP ratio. Any change in ATP level is then corrected for by a feedback type of response which alters the ratio of substrate oxidized to substrate used for anabolism.

Apparently a high level of ATP favors anabolism and a low level favors catabolism.

Responses of this type under reduced DO concentration, however, are not the usual case reported for the wide majority of studies on oxygen-linked metabolism by various microorganisms. The most predominant observation is that respiration and metabolism proceed independent of DO until very low levels are reached. Decreases in oxygen concentration below this critical level then result in a slowing of metabolic reactions.

Button and Garver (8) found the affinity constant (K_s) for oxygen of a chemostat culture of Torulopsis utilis growing on glycerol to be 0.45 ppm 0_2 (1.4×10^{-5}M). Later work by Johnson (46) on Candida utilis grown on acetate indicated a K_m for oxygen of 0.04 ppm (1.3×10^{-6}M). The results of other workers (6) are in good agreement with the findings of a low oxygen requirement in this yeast. Saccharomyces cerevisiae has been shown to carry out oxidative metabolism at levels as low as 0.06 - 0.12 ppm 0_2 (5, 73).

Carter and Bull (11, 12) used an automatic DO control system (53) in continuous cultures of Aspergillus nidulans growing on glucose and found that the critical level for this fungus was 0.08 mg/l DO at 30°C (1.75 mmHg).

Below this level nitrite accumulated in the medium and changes in morphology were noted.

Harrison, Maclennan and Pirt (40) studying continuous cultures of Pseudomonas DX2, and Maclennan and Pirt (54) using the same organism under automatically controlled DO, determined the growth limiting or "critical" DO concentration was 0.04 - 0.3 mg/l DO at 30°C (1.0 - 7.0 mmHg) for decane as substrate and < 0.04 mg/l with glucose as the carbon source. Their interpretation of the data suggested that for growth of this organism on glucose, two oxygen uptake enzyme systems or two states of the same system were involved. The reaction dominating at low DO below 0.17 mg/l DO (4.2 mmHg) would have an increased affinity for oxygen with an apparent Km of only 0.01 mg/l DO (0.2 mmHg). For growth on decane they found that below the critical level respiration rate decreased with an increase in decane utilization occurring around 0.12 mg/l DO (3 mm Hg). An increase in the formation of extracellular products from the incomplete oxidation of decane was detected at this point. The authors deduced that at low DO there was either an increase in the amount of oxygenase in the cell or that there was some release of inhibition on the enzyme.

Maclennan et al (55) employed essentially the same methods as previous workers (54) on continuous cultures of the strict aerobe Pseudomonas AM1. The critical DO for growth on methanol was 0.28 mg/l DO at 30°C (7 mmHg). Below this value the culture became oxygen-limited and residual quantities of methanol increased.

For the facultative anaerobe Beneckea natriegens respiration and glucose oxidation were recently (49) found to be independent of DO down to 0.08 mg/l DO at 30°C (2 mmHg). Only when the oxygen supply rate fell behind the oxygen demand rate was there a switch to fermentative metabolism.

Studies on the oxygen requirements of species of aerobic psychrotolerant Pseudomonas and Achromobacter have shown that the oxygen concentration of the suspending medium could be lowered to 0.17 mg/l DO at 22°C (2% saturation) without noticeably inhibiting growth rate (18).

It is of interest to note that in many instances (12, 18, 92) when microorganisms are grown under low oxygen concentrations an adaptive response in the form of a decreased requirement for oxygen (lowered critical DO) becomes apparent. The synthesis of cytochromes by both obligate aerobes and facultative organisms is

strongly influenced by the environment, and in particular, by the degree of aeration. Many reports of maximum respiratory enzyme content under low DO concentration have appeared in the literature (20, 40, 61, 62, 63, 75, 77, 80). Meyer and Jones (59, 60) estimated the efficiency of energy production via oxidative phosphorylation in several species of bacteria selected on the basis of their cytochrome oxidase composition. In summarizing their results they concluded that, at low levels of DO, oxidases associated with respiratory chains of low energy conservation efficiency were synthesized in greater amounts; that oxidases with increased affinities for oxygen became operative so that energy production could proceed, although inefficiently, at very low oxygen concentrations was yet another possibility.

Information on the response of mixed cultures to DO concentration is of practical importance in the field of water pollution control. The three basic methods presently employed in waste treatment are the trickling filter (7, 58), the activated sludge process (15, 82), and the oxidation or stabilization pond (68). One of the more recent innovations is the aerated lagoon (57, 76), a system initially developed to supplement the supply of oxygen during the period of spring break-up, and to alleviate nuisance odours by artificially aerating stabilization ponds. Although specialized engineering structures and

mechanical equipment for the variations in these methods vary widely, the fundamental process of aerobic biological decomposition of organic wastes remains basically unchanged from system to system.

A sewage lagoon might best be described as a continuous-flow enrichment culture of microorganisms. A wide variety of microbial types are present in mixed culture with the predominating species being determined by the characteristics of the waste input and environmental influences such as climate and operational procedures. It is known that bacterial numbers, activities and biotic types vary in response to seasonal changes (36) and other parameters (85). In a lagoon system several factors mediate the amounts and types of substrates present but the overall homogeneity of the organic loading in lagoons serving domestic communities results in a fairly constant substrate supply. The pH in this instance has been shown to fluctuate only moderately (36).

Two of the most important physical factors affecting the efficiency of aerobic wastes stabilization would appear to be temperature and oxygen supply. The efficiency of a waste treatment process is usually reported as the percent removal of biochemical oxygen demand over a five-day test period at 20°C (BOD_5). Temperature

has been shown to effect BOD_5 reduction in varying degrees for activated sludge units, stabilization ponds and aerated lagoons (19, 22, 34, 70). Vennes and Olson (90) found no difference in BOD_5 reduction between 0^oC to 20^oC in an aerated lagoon operating with a detention time of 40 days under much the same conditions as the lagoon described in the present study. They concluded that the limiting parameter in such a system would be delivered or biologically utilizable oxygen.

While it is generally assumed that the concentration of dissolved oxygen plays a vital role in the purification of wastes, relatively little information is available on the actual biological requirement for oxygen in the breakdown of organics. Much of the applied work done to date has been directed towards the activated sludge process where the microorganisms responsible for stabilization grow in flocculated masses. In this system the amount of oxidation is dependent on the diffusion of oxygen into the microbial floc.

Mueller et al (65) have reported that for Zoogloea ramigera floc particles, the DO concentration that would become limiting for respiration was in the range 0.6 - 2.5 ppm and was dependent on the size and activity of the floc. They also found that the critical oxygen concentration for dispersed cells of the same organism was below 0.1 ppm DO. The authors suggested that for most

activated sludge plants operating at conventional mixing levels, DO concentrations below 2 ppm would not affect substrate removal and that in many cases, where higher degrees of agitation are used, the oxygen concentration could be lowered to 0.6 ppm DO without any serious effect on efficiency. Other workers (29, 87) have maintained that the critical level would lie in the region below 0.5 ppm DO. Englande and Eckenfelder (24) found that in a laboratory-scale activated sludge unit the rate of phenol removal decreased when the DO was lowered below a critical level of 0.5 ppm.

In a sewage lagoon the indigenous microflora are in a dispersed state of growth so that the diffusion of oxygen to individual cells is in all probability not as critical a factor as in other processes. It appears, however, that no work has been done on the oxygen requirements of mixed bacterial populations indigenous to a lagoon operation.

From the literature it would seem that fairly low levels of DO might be permissible. Critical dissolved oxygen values have ranged from 0.1 to 1.53 ppm DO for various pure cultures and 0.2 to 0.5 ppm DO for activated sludge (24). Recommended minimum operating DO concentrations in activated sludge plants have ranged from 0.5 -

2.0 ppm (87). The minimum operating level for the aerated systems in the Charleswood Lagoon, Winnipeg, Manitoba is set at 2.0 ppm DO (71).

When as complex a system as a sewage lagoon is used as a source of microorganisms for laboratory studies it would be beneficial to have a relatively simple method to correct for fluctuations in biomass. The method of choice should use as a reference some measureable variable that depends on metabolic activity and viable biomass. It should also reflect the rate of substrate removal.

Classical methods for estimating bacterial mass and numbers such as the standard plate count, dry weight, and optical density measurements provide little or no information on biochemical activity. Coulter counter techniques (36) do not adequately correct for suspended particulate materials in making direct counts on bacterial numbers. Endogenous respiration rate (O_2 uptake) determined by Warburg manometry has been used as a measure of bacterial concentration in sewage (23), but this does not always predict the potential activity in the presence of substrate. Organic nitrogen (86) and DNA content (91) have been proposed as estimates of cell population but these parameters do not seem to be a good estimate of cell viability. Dehydrogenase enzyme activity, assayed

by the reduction of tetrazolium salts (eg. TTC) to give a colorimetric response, has been used as a measure of general biological activity (31). Measurement of ATP pools in heterogeneous microbial populations has also been investigated as an indication of sludge activity (17, 26). No acceptable single standard procedure for estimating viable biomass of mixed cultures has been put forward.

METHODS AND MATERIALS

METHODS AND MATERIALS

Description of the Charleswood Lagoon

The Charleswood Lagoon (71) is located at approximately 50^{o}N latitude in a region where the climate is of the mid-continental type. The mean ambient air temperature during the summer is $+19^{o}$C and during the winter -27^{o}C.

The general layout of the lagoon is shown in Fig. 1 and a schematic of the aerated system is shown in Fig. 2. The average design flow of the conventional cells is 3×10^{6} gallons domestic waste materials per day. The aerated system consisting of Air Aqua, Surface Aerator and Air-Gun installations has an average flow of 0.5×10^{6} gallons per day. In the Air Aqua cells air is delivered from a compressor to plastic aeration tubing set on the bottom of the basin in a grid pattern. Openings in the tubing allow air bubbles to be released upon which oxygen transfer occurs as the bubbles rise to the surface. The Surface Aerator installation consists basically of eight aerators placed in series. Oxygen transfer is accomplished by the spinning action of the impeller blade at the liquid

Fig. 1. Salient features of the Charleswood Lagoon.

A - Primary cell

B - Secondary cell

C - Air-Gun

D - Surface Aerator

E - Air Aqua cell

F - Summer sampling site

G - Winter sampling site

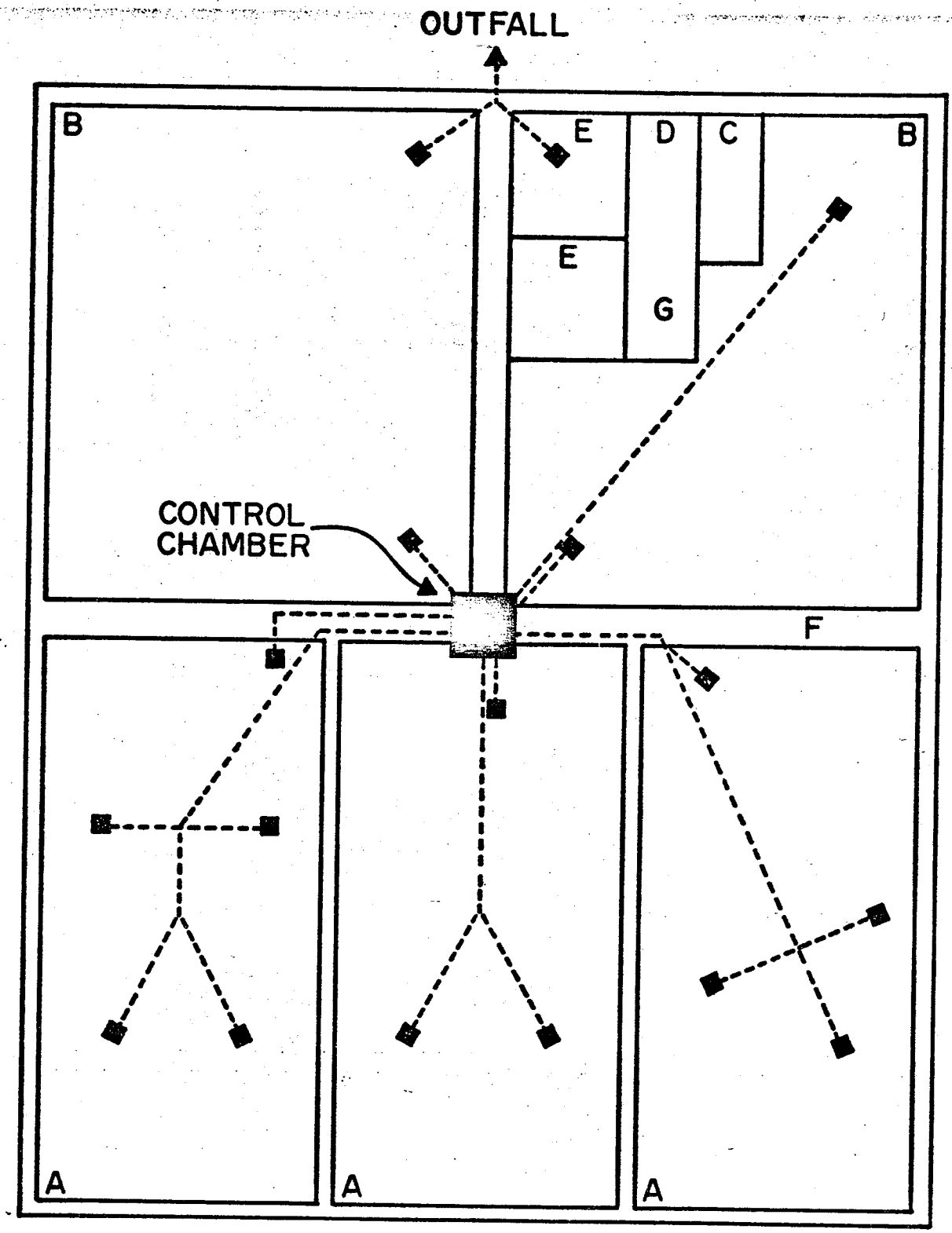
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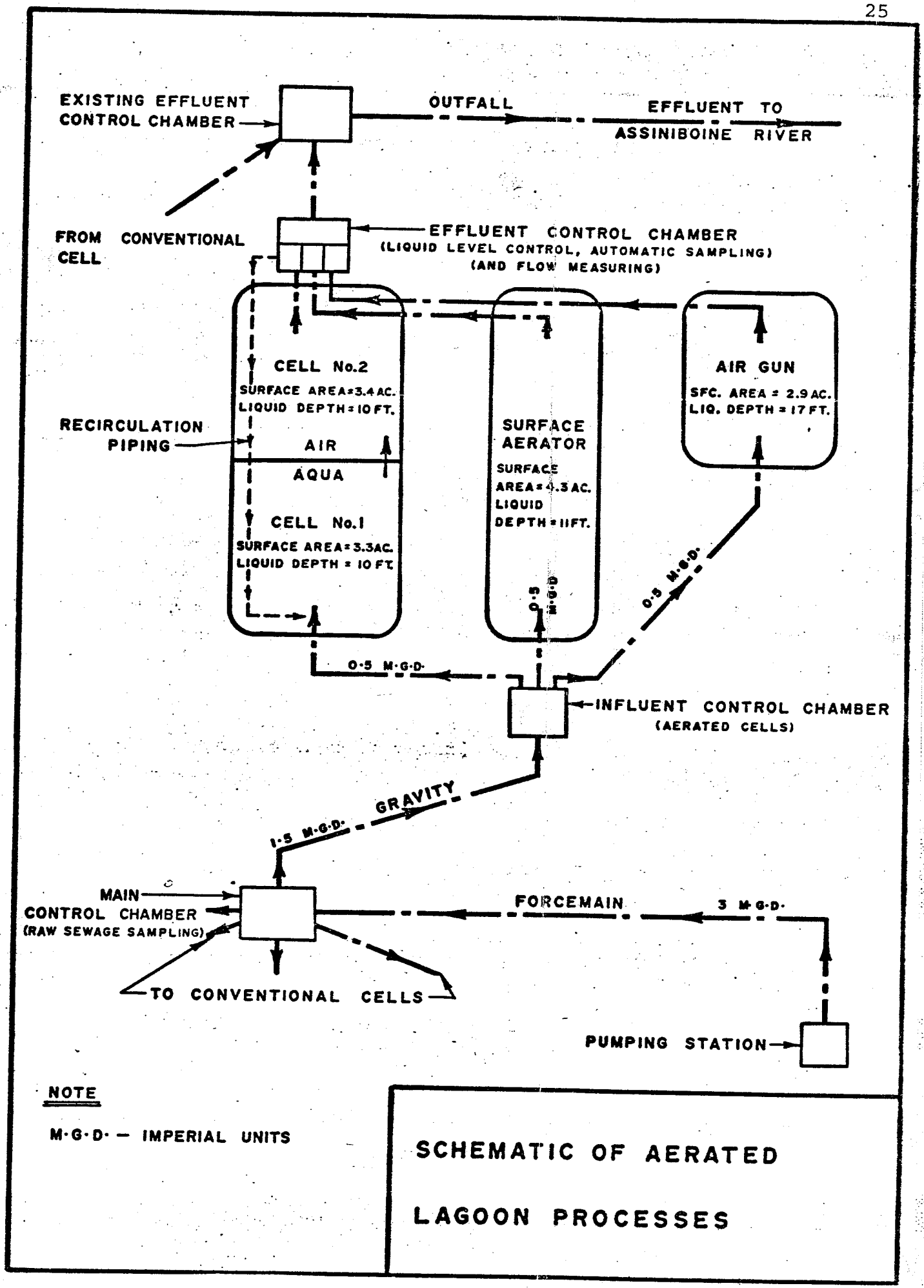
Fig. 2. Schematic illustrating the flow pattern for operation of the aerated installations in the Charleswood Lagoon.



NOTE

M·G·D· — IMPERIAL UNITS

SCHEMATIC OF AERATED LAGOON PROCESSES

surface. This creates a multitude of air bubbles and at the same time tosses the liquid into the air. The Air-Gun system delivers air from a compressor to 54 air-guns placed in a grid pattern in the lagoon cell. Oxygen transfer is effected by a large air bubble formed inside the gun which rises to the surface and explodes agitating the surface violently. In the conventional cells oxygen is introduced by algal photosynthesis and by natural exchange with the atmosphere at the air-water interface. Under cold winter conditions both of these oxygen sources in conventional cells are negated by the formation of an ice-cover.

Sampling Procedure

Lagoon water was collected from the Charleswood Lagoon during the summer of 1973 and winter of 1974. During the summer period lagoon water was taken from a permanent sampling port located at the effluent end of the Primary No. 1 conventional lagoon cell using a Briggs and Stratton four-cycle centrifugal pump. Under winter conditions samples were withdrawn manually from the platform housing the surface aerator assembly. The bacteria were recovered from these samples and their biochemical activity was estimated.

Preparation of Resting Cell Suspensions

A mixed bacterial population indigenous to the lagoon was employed to study the effect of reduced dissolved oxygen concentrations on substrate stabilization. Resting cell suspensions were prepared by a modified version of the method developed by Halvorson et al (36).

The organisms present in a sample of lagoon water (130 to 140 liters) were collected by centrifugation using a steam driven Sharples Super Centrifuge (Sharples Centrifuges Limited, Camberly, Surrey) operating at 20 lbs. steam pressure (approx. 40,000 rpm). Cell losses were judged to be minimal upon microscopic examination of the supernatant liquid. The packed sediment containing algae, protozoa, bacteria and particulate deposits was washed from the rotor with 50 mM potassium phosphate buffer pH 7.0 and placed on a magnetic stirrer to break up clumps of organisms and resuspend individual cells. Algae, protozoa and detritus were separated from the bacteria by low speed differential centrifugation at 2,000 rpm (650 x g) for 10 minutes using a model RC2-B Sorvall Superspeed centrifuge (Ivan Sorvall Inc., Norwalk, Conn.). The supernatant liquid containing the bacteria was decanted and placed back on a magnetic stirrer. The sediment was washed in buffer once to

free trapped bacteria and re-centrifuged at low speed. The combined supernatant liquids were then centrifuged at 6,000 rpm (5,800 x g) for 15 minutes and the top 70 to 80% of the supernatant liquid was discarded. This was done in an attempt to rid the preparation of light fatty materials originally present in the lagoon water. The remainder was resuspended in buffer and spun at 2,000 rpm (360 x g) for 10 minutes to remove algae carried over from the previous separation. The supernatant liquid containing the bacteria was then carefully decanted into another centrifuge bottle and spun at 10,000 rpm (16,300 x g) for 15 minutes. The sedimented bacteria were washed in buffer again to remove any residual soluble compounds, spun down at high speed and resuspended. The required volume of 50 mM phosphate buffer was then added to the suspension to arrive at a 100 fold concentration of the bacterial population existing in the lagoon water at the time of sampling. The number of bacteria originally present in 1 liter of lagoon water would be equivalent to 10 ml of cell preparation. The cells were then placed on a magnetic stirrer and allowed to respire overnight to remove traces of nutrient not extracted in the washing process and to reduce the endogenous rate of metabolism by depleting cellular energy reserves.

The above procedure was carried out at ambient room temperature in the case of summer mesophilic preparations and at 10°C for winter psychrotrophic bacterial suspensions.

The biomass was estimated by a manometric procedure to be described. Cell suspensions for this procedure were prepared by removing a portion of the final cell preparation described above, centrifuging at high speed and resuspending the bacteria in the same phosphate buffer to give a bacterial concentration of 1000 X that present in the original sample of lagoon water.

In experiments utilizing linear alkylbenzene sulfonate (LAS) detergent as substrate, bacteria were suspended in distilled water rather than in phosphate buffer due to interference produced by inorganic phosphates in the quantitative analysis of anionic surfactants by the "methylene blue" method (81). Suspensions for manometric determinations were still made up with buffer solution.

Measurement and Control of Dissolved Oxygen in Bacterial Resting Cell Suspensions

Maintenance of a desired dissolved oxygen concentration in stirred resting cell suspensions was achieved by an automatic control system modified after

Maclennan and Pirt (53), which used a Mackereth oxygen electrode as the primary sensing element. The equipment assembly is pictured in Fig. 3.

The dissolved oxygen concentration in a cell suspension was measured by a model A15A biological electrode in conjunction with a model 15A dissolved oxygen meter equipped with a combined temperature compensator and resistance thermometer probe which corrects for temperature effects on DO readings (Electronic Instruments Ltd., Richmond Surrey, Eng.). The electrode is a lead-silver galvanic cell sheathed in a polythene membrane which is permeable only to oxygen dissolved in the surrounding medium. The cell generates a current due to the chemical reduction of oxygen diffusing through the membrane which is directly proportional to the oxygen partial pressure in solution. This output current is fed via an external DO meter connection to a Series 60 3-Action Current-Adjusting Type (C.A.T.) control unit equipped with a Speedomax H recorder (Leeds and Northrup, Sunneytown Pike, North Wales, Pa.). The recorder-control unit compares the signal from the oxygen sensing electrode with a pre-set reference position corresponding to the desired dissolved oxygen concentration. The control unit gives out a corrective signal

Fig. 3. Dissolved oxygen control system assembly.

A - Constant temperature circulator

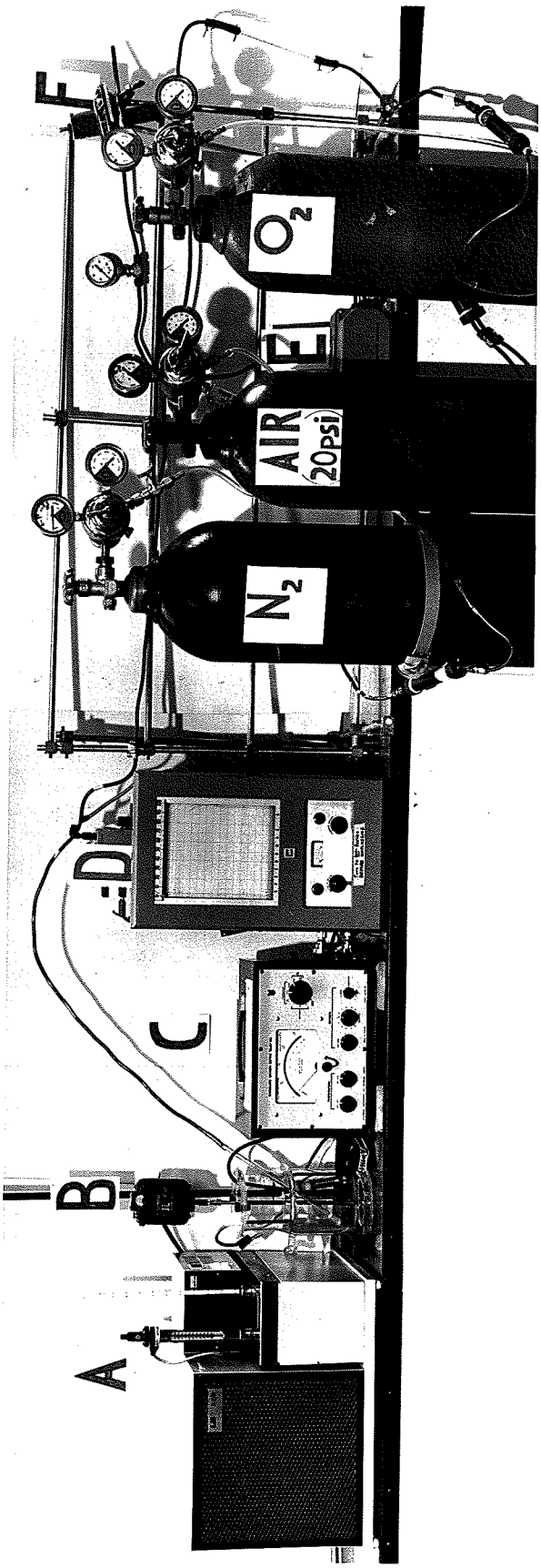
B - Water-jacketed reaction chamber containing dissolved oxygen meter probes

C - Dissolved oxygen meter

D - Recorder-controller

E - Pneumatic converter

F - Control valve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O_2

varying between 1 and 5 mA. dependent upon the deviation of dissolved oxygen from the control point and rate of change of the deviation. This signal is then fed to a model A electro-pneumatic converter (Leeds and Northrup, Sunneytown Pike, North Wales, Pa.) which converts it to a corresponding output air pressure between 3 and 15 lbs/sq inch. The output pressure operates a Pneumatic Control Valve (Research Control Valves, Tulsa, Okla.) which in turn regulates the amount of oxygen admitted to a constant nitrogen gas stream flowing to the resting cell suspension.

The oxygen control equipment was fitted to a 1 liter water-jacketed jar in which the microbial suspension was placed and stirred at a rate of 1,000 rpm by a 1/2 hp variable speed electric motor (Palo Laboratory Supplies, New York, N. Y.). Temperature of the suspension was controlled to within ± 0.02°C by a Lauda constant temperature circulator (Lauda Instruments Division, Brinkman Instruments Inc., Westbury, N. Y.).

The system outlined controlled DO levels to within 1% of the required oxygen concentration over the range from 0.24 to 2.0 ppm resulting in a maximum error of ± 0.08 ppm at 25°C and ± 0.13 ppm at 2°C.

Aerobic and Anaerobic Control Systems

Complete aerobiosis of resting cell suspension controls was accomplished by using a Precision Warburg Manometrician apparatus (Precision Scientific Co., Chicago Ill.) as a convenient temperature controlled waterbath in which the bacterial suspensions could be agitated. The apparatus was equipped with both a heating and cooling coil which controlled the temperature to within ± 0.02°C. For aerobic controls a 50 ml volume of the cell suspension was placed into a 125 ml Warburg flask and attached to the manometer so that the flask contents were open to the atmosphere. Flasks were shaken at a rate of 100 oscillations per minute to ensure an adequate supply of oxygen.

The anaerobic control system was simply a serum bottle containing 50 ml of the bacterial suspension which was sealed with a serum stopper. Anaerobic conditions were created by thoroughly flushing the serum bottle with a flow of nitrogen gas after substrate addition and keeping it in a static position in the same waterbath as the aerobic control.

Substrates

Materials selected as substrates for this investigation were all organic compounds known to require

molecular oxygen for their microbially mediated breakdown. A total of seven substrates was tested. The representative detergent used was a commercial linear alkylbenzene sulfonate (LAS) with an average chain length of 11.3 carbons, prepared by sulfonation of a linear alkylate having 11 carbon atoms with the phenyl group attached to the number 3 carbon (a gift of Dr. R. D. Swisher, Monsanto Chemical Co., St. Louis, Mo.). The short chain fatty acids acetate, propionate and butyrate, along with benzoate phenol and ethanol were also employed. All of these compounds were of the highest grade commercially available.

Standard Experimental Procedure

All experimental work, whether dealing with mesophilic or psychrotrophic organisms, conformed to the following protocol: A volume of lagoon water serving as the source of bacteria was collected from the Charleswood Lagoon installation and a resting cell suspension (100 x concentration) was prepared as previously described (pg.27). A portion of this suspension was taken, concentrated to a 1,000 x concentration by centrifugation and resuspension, and stored under refrigeration until the following day when oxygen uptake was measured by manometry (see pg.41). The dissolved oxygen electrode and meter were calibrated at the operating temperature

to be used with air-saturated distilled water in order to provide accurate monitoring of oxygen concentration during the course of the experiment. The resting cell suspension (100 x) was then placed into the water-jacketed reaction chamber and the DO control system was set into operation. At the same time a 50 ml volume of the suspension was placed into a 125 ml flask open to the atmosphere and the flask was set into the Precision Warburg waterbath operating at the same temperature as the controlled DO setup. This flask served as an aerobic control and was taken to represent the optimum metabolic rate of substrate utilization with respect to DO concentration. An anaerobic control was also included to establish with confidence that the test compound was not being dissimilated in the absence of oxygen by a fermentative pathway. After an approximate one hour period, during which temperature was equilibrated and oxygen concentration was stabilized, substrate was added to the suspensions to give a final concentration of approximately 100 ppm. Samples were taken at zero time and at suitable time intervals throughout the span of the experiment, the cells were removed by centrifugation at 12,000 rpm (17,300 x g) for 10 minutes in the Sorvall centrifuge, and the supernatant liquid was frozen and stored for subsequent

analysis of residual substrate. Anaerobic samples were taken with a hypodermic syringe after which the serum bottle was flushed with nitrogen to ensure anaerobiosis.

The term critical DO level as used here will refer to that dissolved oxygen level above which the rate of substrate utilization is independent of oxygen concentration and below which the rate of degradation is reduced. Critical levels were determined by comparison of an aerobic trial with a trial performed simultaneously at a reduced DO concentration using the same bacterial resting cell suspension. Trials were carried out at 25°C in the case of mesophilic bacteria and at 2°C for psychrotrophic organisms. Aerobic controls would represent a dissolved oxygen concentration (assuming saturation) of 8.1 ppm at 25°C and 13.4 ppm at 2°C under one atmosphere air pressure. In any given experiment oxygen supply was the only variable imposed.

Analytical Methods

The quantitative analysis of LAS detergent was done by the standard "methylene blue" method (81). This method is based on the formation of a blue-colored salt when methylene blue reacts with anionic surfactants. The salt is solubilized in chloroform and the intensity of color, measured by a Klett-Summerson photoelectric

colorimeter (Klett Mfg. Co. Inc., New York, N. Y.), is proportional to the detergent concentration. A colorimetric calibration curve (Fig. 5) prepared from tetrapropylene alkylbenzene sulfonate (ABS) served as the reference standard. Materials determined by this method are frequently designated "methylene blue" active substance (MBAS).

All other quantitative analyses were done by gas chromatographic techniques utilizing a Varian Aerograph Model 2100 gas chromatograph (Varian Aerograph, Walnut Creek, Cal.) equipped with a flame ionization detector. The carrier gas was nitrogen and the flame was supported by a constant flow of compressed air (300 cc/min) and hydrogen (35 cc/min). A 10 microliter syringe (Glenco Scientific, Inc., Houston, Texas) was used for "on column" injection of samples.

Acetate, propionate, butyrate, ethanol and phenol were all estimated by gas-solid chromatography using a 1.83 m by 3 mm internal diameter glass "U" column packed with Chromosorb 102, 80/100 mesh (Johns-Manville, Celite Div., Denver, Co.). Chromosorb 102 is a high surface area solid adsorbent which permits direct analysis of aqueous organic solutions of the type encountered in this study.

Benzoic acid was estimated by gas-liquid chromatography using a 1.83 m by 3 mm internal diameter glass "U" column packed with Gas-Chrom "Q", 60/80 mesh, treated with a three percent (3%) OV-1 coating as the liquid phase (Applied Science Laboratories Inc., State College, Penn.). Samples containing benzoate, which had been collected and frozen during an experiment, were lyophilized to remove water (78). Solid benzoate was resolubilized and the trimethyl silyl derivative prepared by the addition of 1.0 ml of Tri-Sil (Pierce Chemical Co., Rockford, Ill.). Samples were then stored at room temperature in air tight plastic stoppered glass vials for 24 hrs to ensure that derivitization was complete prior to gas chromatographic analysis.

The various operational characteristics employed for gas chromatographic analyses are given in Table 1. The respective concentrations of compounds undergoing gas chromatographic analyses were determined by measuring the total area under the resultant peak of the sample injection with the aid of a disk-type integrator.

TABLE I

Operational characteristics of gas chromatographic quantitative analyses

Compound	Carrier gas flow (cc/min)	Temperatures (^{o}C)			Sensitivities	
		Injector	Column	Detector	Attenuation	Range (amp/mv)
Acetate	31	220	195	220	64	10^{-12}
Propionate	39	220	195	220	128	10^{-12}
Butyrate	48	220	200	220	128	10^{-12}
Ethanol	28	175	150	175	128	10^{-12}
Phenol	65	240	220	240	16	10^{-11}
Benzoate	31	155	110	160	256	10^{-12}

Manometric Standardization of Active Biomass in Bacterial Resting Cell Suspensions

Exogenous oxygen uptake was chosen as the criterion for the estimation of active biomass in mixed bacterial cell suspensions for comparative purposes. A Bronwill Warburg instrument (Bronwill Scientific Inc., Rochester, N. Y.) was used to measure oxygen uptake. Standard manometric techniques with air as the gas phase were utilized (88). A resting cell suspension of 1,000 x concentration was used. Each flask contained 1.0 ml of cell suspension in the main compartment. A small fluted filter paper and 0.2 ml of a 20% (w/v) KOH solution was placed in the center well to absorb CO_2 produced. The side arms held 0.8 ml of a 0.75% (w/v) vitamin-free casamino acids solution (Difco Laboratories, Detroit, Michigan) serving as substrate. No substrate was added to endogenously respiring controls. The total fluid volume of each flask was brought up to 3.2 ml with 50 mM potassium phosphate buffer pH 7.0. Flasks were then attached to manometers, placed in the Warburg thermoregulated waterbath, and shaken at a rate of 60 oscillations per minute. After a 20 minute period of temperature, gas and liquid phase equilibration, substrate was tipped

into the respiring cell suspension. Manometer readings were taken at 10 minute intervals for a period of 60 to 90 minutes. Data obtained were used to calculate microliters of oxygen consumed in one hour by 1.0 ml of bacterial suspension utilizing vitamin-free casamino acids substrate. One standardized unit of activity for the biomass correction is defined as being equivalent to an exogenous oxygen uptake of 1.0 µl O_2 hr^{-1} ml^{-1} of cell suspension designated as A_{ca} (activity on casamino acids).

Manometric oxygen uptake involving mesophilic preparations was carried out at 30°C while uptake involving psychrotrophic organisms was performed at 15°C.

Assessment of Manometric Standardization of Active Biomass

If the manometric method is to provide a reliable estimate of bacterial activity in mixed cultures the rate of substrate (eg. acetate) removal per standardized unit of biomass activity (A_{ca}) should remain constant from one resting cell preparation to another. To determine if this was the case the test substrate was added to a cell suspension and the same methodology followed as previously described for the aerobic controls (pg. 36). The exogenous oxygen uptake of the suspension was then

measured as outlined above. The rate of substrate removal was determined by measuring the slope of the time-course utilization curve at the maximum rate of degradation. A biomass correction was then carried out by calculating the rate of substrate removal per standardized unit of bacterial activity as measured by manometry. The same procedure was followed for the utilization of propionate, butyrate, ethanol, phenol, benzoate and LAS by a number of cell suspensions prepared at different times. The aerobic control samples employed with controlled DO trials were also included in the calculations. It should be noted that the time-course substrate utilization curves presented in the Results section are not graphically corrected for fluctuations in active biomass associated with lagoon water sampled at different times.

In one experiment of the type just outlined all of the substrates, with the exception of LAS, were added in combination to a cell suspension to determine if the rate of utilization of any or all of the compounds concerned was affected by a multiple substrate condition i.e. if any diauxic phenomena were occurring.

RESULTS

RESULTS

I. Standard Curves for Quantitative Analyses

A composite standard curve depicting the linear recorder response for the injection of materials within the range 0 to 1.0 µg for the determination of acetate, propionate, butyrate, ethanol and phenol by gas-solid chromatography is shown in Fig. 4a. Under the respective operational characteristics employed (see Table I) the retention times were as follows: acetate, 4.2 min; propionate, 6.9 min; butyrate, 12.2 min; ethanol, 5.6 min; phenol, 19 min.

Fig. 4b shows the standard curve for the quantitative estimation of benzoate by gas-liquid chromatography using the trimethylsilyl derivative. The linear recorder response is shown for benzoate injections within the range 0 to 3.0 µg. Under the operational characteristics employed (see Table I) the retention time was 9.8 minutes.

The standard curve for the colorimetric determination of LAS detergent measured as "methylene blue active substance" (MBAS) using the red filter is shown in Fig. 5.

Fig. 4. Standard curves obtained by gas chromatography for the quantitative analysis of a) acetate (■), propionate (□), butyrate (△), ethanol (▲), and phenol (●) and b) benzoate (o).

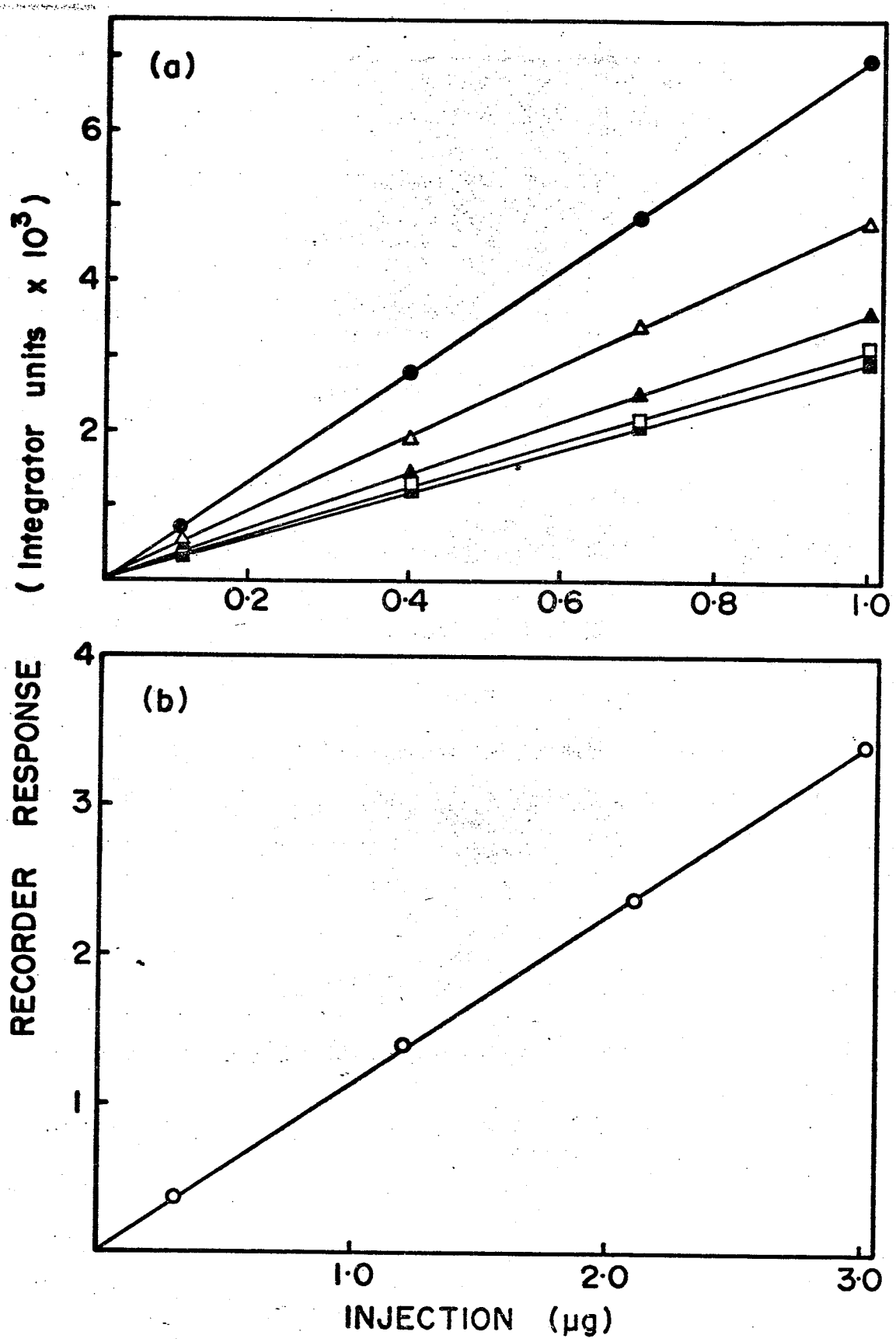
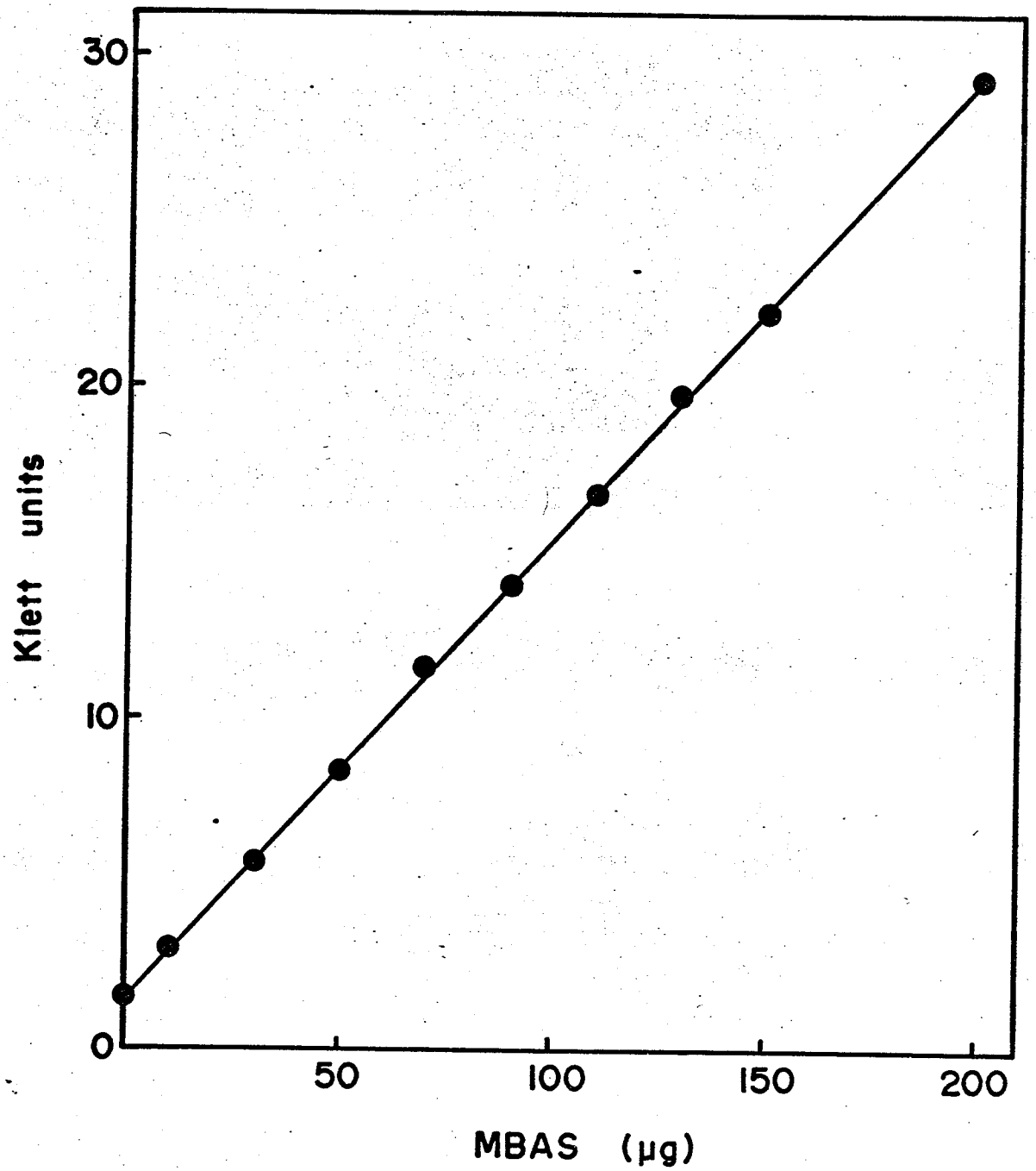

(a)
(Integrator units x 10^3)
6
4
2
0·2
0·4
0·6
0·8
1·0
(b)
RECORDER RESPONSE
4
3
2
1
1·0
2·0
3·0
INJECTION (µg)

Fig. 5. Standard curve obtained by colorimetry for the quantitative analysis of LAS as "methylene blue active substance" (MBAS).


30
20
10
0
Klett units
50
100
150
200
MBAS (µg)

II. Utilization of Selected Organic Substrates by Mesophilic Sewage Lagoon Bacteria at 25°C as a Function of Dissolved Oxygen Concentration

A) LAS

LAS was degraded under aerobic conditions by mesophilic lagoon bacteria. The rate of utilization was not affected by a dissolved oxygen level as low as 0.24 ppm DO. LAS was not metabolized anaerobically.

The time-course curves for LAS degradation by mesophiles under 0.5 ppm and 0.24 ppm DO together with the respective aerobic and anaerobic controls are shown in Figs. 6a and 6b.

B) Phenol

It was found that phenol was utilized under aerobic conditions by mesophilic lagoon bacteria. Under anaerobic conditions no breakdown of phenol was observed. Lowering the oxygen concentration to 0.24 ppm did not alter the rate of phenol removal. There was an approximate 8 to 10 hour lag period before degradation was initiated and the rate of breakdown decreased considerably after 55 hours. The degradation curve for the utilization of phenol under aerobic, anaerobic and 0.24 ppm DO is shown in Fig. 7.

Fig. 6. Utilization of LAS at 25°C (under a) 0.5 ppm DO (◓) and b) 0.24 ppm DO (◓) by resting cell suspensions of mesophilic lagoon bacteria along with corresponding aerobic (○) and anaerobic (●) controls.

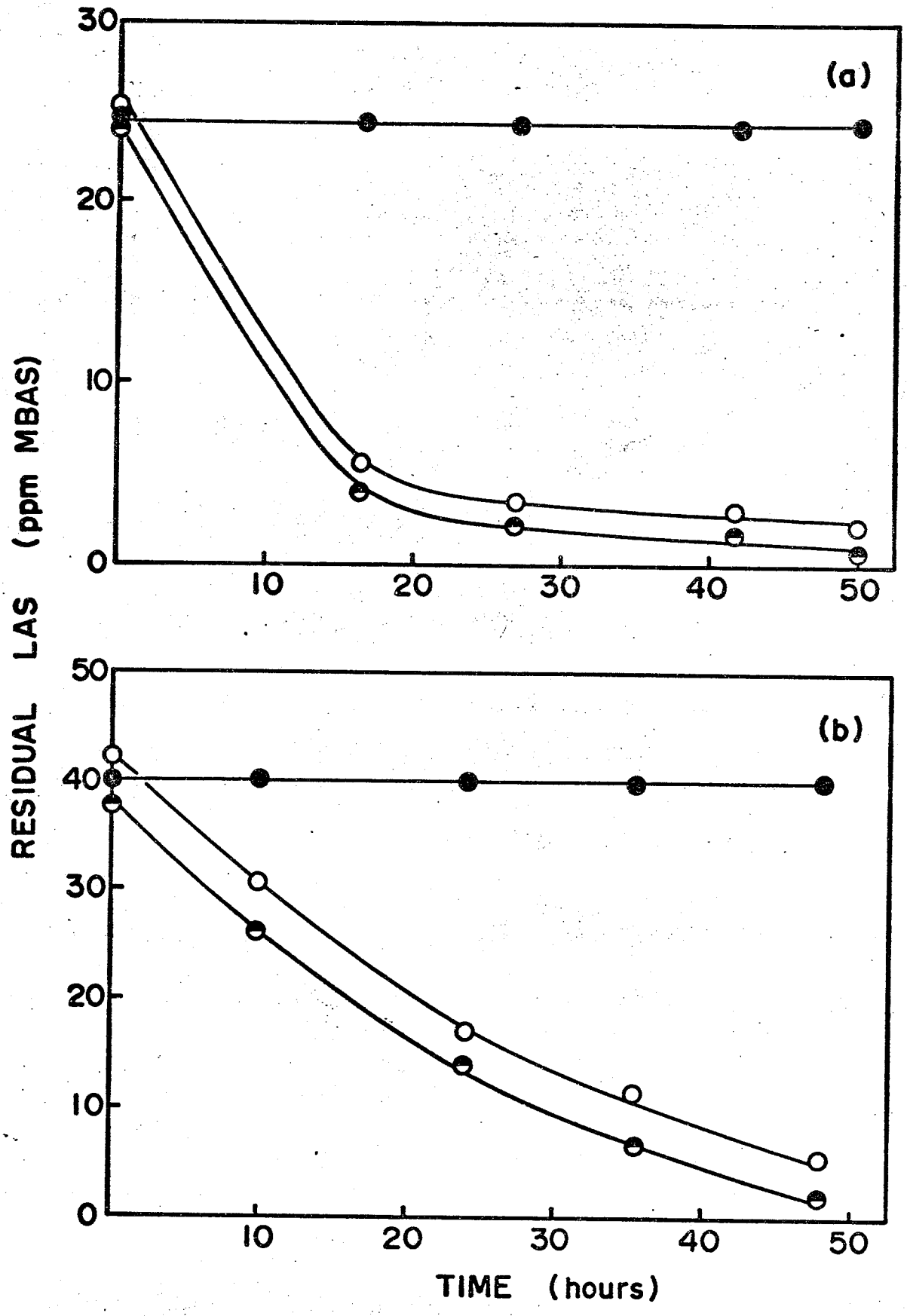
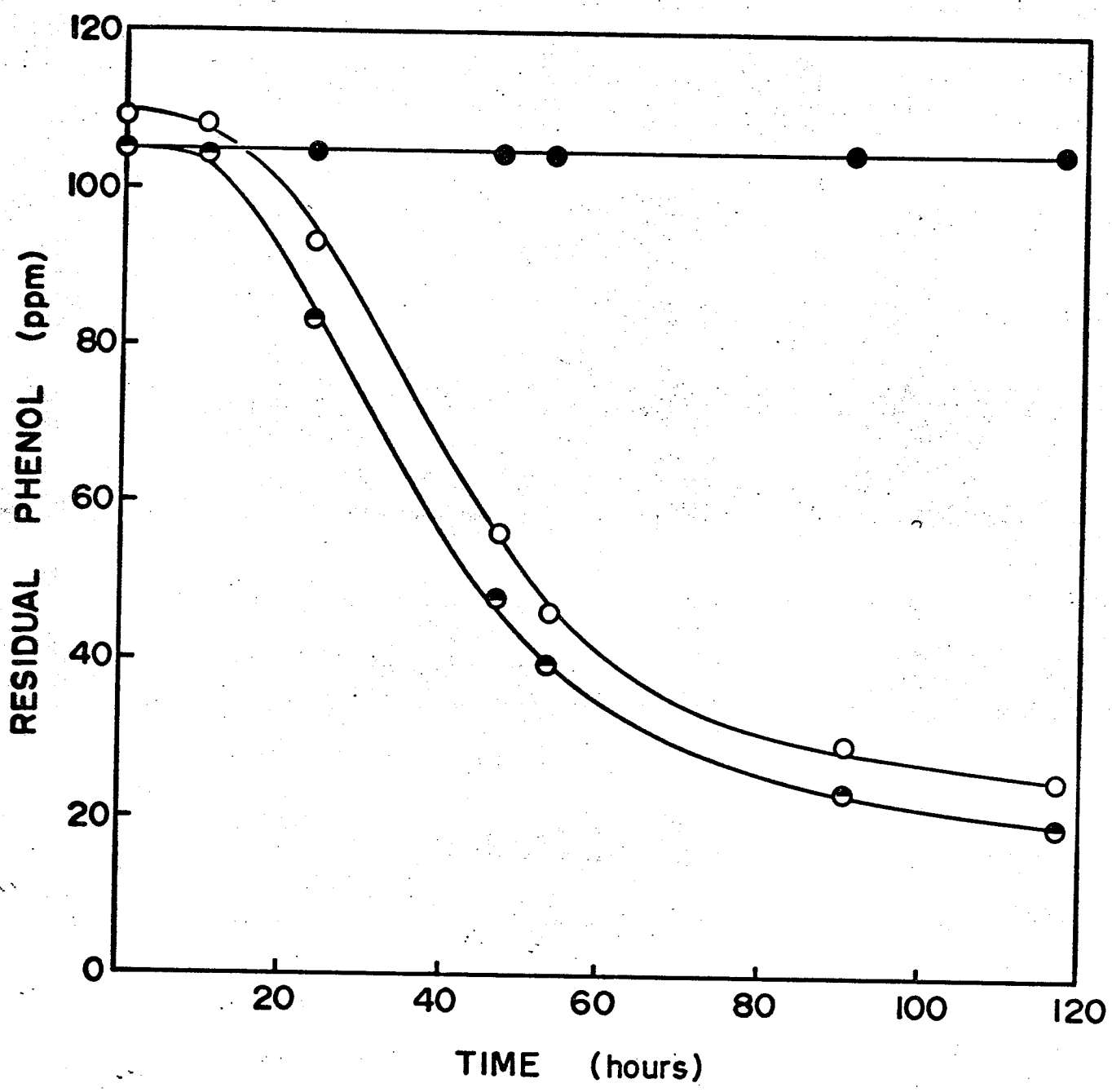

(a)
(b)
RESIDUAL LAS (ppm MBAS)
TIME (hours)
0
10
20
30
40
50

Fig. 7. Utilization of phenol at 25°C under 0.24 ppm DO (◒) by a resting cell suspension of mesophilic lagoon bacteria along with aerobic (O) and anaerobic (●) controls.


120
100
80
60
40
20
0
RESIDUAL PHENOL (ppm)
20
40
60
80
100
120
TIME (hours)

C) Acetate

Acetate was utilized aerobically by the mesophilic lagoon population employed. Acetate was not used anaerobically. A level of 0.24 ppm DO did not retard the rate of acetate metabolism compared to the rate under fully aerobic conditions.

Curves for the utilization of acetate at 1.0 ppm, 0.5 ppm and 0.24 ppm DO including aerobic and anaerobic controls are shown in Figs. 8a, 8b and 8c.

D) Propionate

Propionate was observed to be utilized under aerobic conditions by mesophilic lagoon bacteria. Under anaerobic conditions no breakdown of propionate occurred.

At a DO level of 2.0 ppm the rate of propionate oxidation was not significantly altered from the rate of the aerobic trial. At 1.5 ppm DO, however, the rate was reduced to 60 per cent that of the aerobic sample. Decreasing the DO to 1.0 ppm resulted in the rate being lowered to 48 per cent that of the aerobic sample. Rates of breakdown of propionate at 2.0 ppm, 1.5 ppm and 1.0 ppm DO along with aerobic and anaerobic controls are shown in Figs. 9a, 9b and 9c.

Fig. 8. Utilization of acetate at 25°C under a) 1.0 ppm DO (◓), b) 0.5 ppm DO (◓) and c) 0.24 ppm DO (◓) by resting cell suspensions of mesophilic lagoon bacteria along with corresponding aerobic (○) and anaerobic (●) controls.

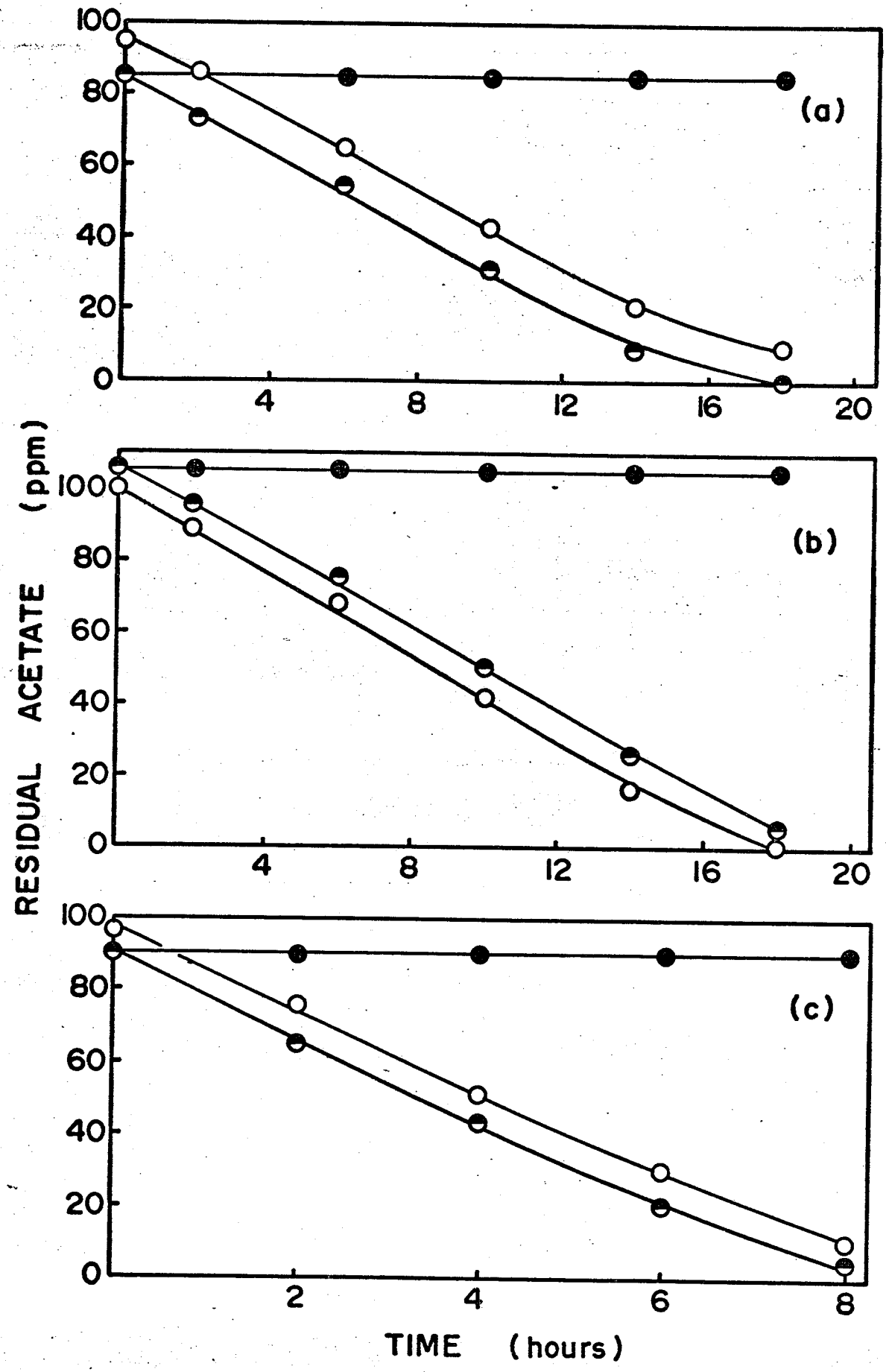
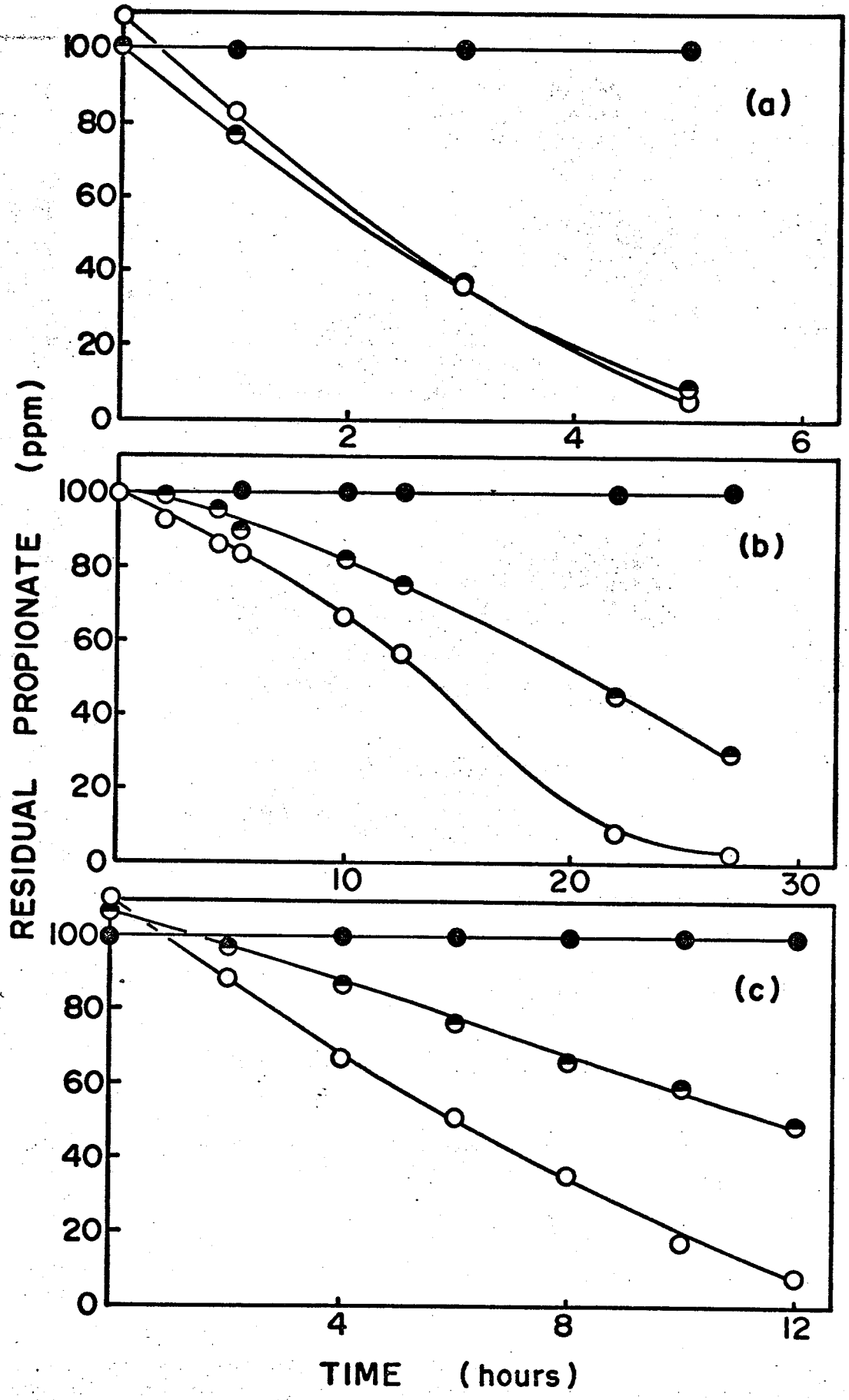

RESIDUAL ACETATE (ppm)
100
80
60
40
20
0
(a)
4
8
12
16
20
(b)
(c)
2
6
8
TIME (hours)

Fig. 9. Utilization of propionate at 25°C under a) 2.0 ppm DO (◓), b) 1.5 ppm DO (◓) and c) 1.0 ppm DO (◓) by resting cell suspensions of mesophilic lagoon bacteria along with corresponding aerobic (○) and anaerobic (●) controls.


RESIDUAL PROPIONATE (ppm)
100
80
60
40
20
0
(a)
2
4
6
(b)
10
20
30
(c)
8
12
TIME (hours)

E) Butyrate

Butyrate was metabolized aerobically by mesophilic bacterial suspensions but not in the absence of oxygen. When the DO was controlled at 1.0 ppm the rate of butyrate utilization appeared to exceed that of the aerobic control. The same result was observed at 0.5 ppm DO. The rates of oxidation of butyrate under aerobic, anaerobic, 1.0 ppm and 0.5 ppm DO levels are depicted in Figs. 10a and 10b.

F) Ethanol

Ethanol was utilized under aerobic conditions but no anaerobic degradation was observed. The rate of utilization at 0.5 ppm DO was identical to that of the aerobic sample. The degradation rate curve for ethanol at 0.5 ppm DO, under aerobic and anaerobic conditions is given in Fig. 11.

III. Utilization of Selected Organic Substrates by Psychrotrophic Sewage Lagoon Bacteria at $2^{\circ}C$ as a Function of Dissolved Oxygen Concentration

A) LAS

LAS was not metabolized by psychrotrophic bacterial cell suspensions under fully aerobic conditions or anaerobically during an 8-day test period. Further

Fig. 10. Utilization of butyrate at 25°C under a) 1.0 ppm DO (◓) and b) 0.5 ppm DO (◓) by resting cell suspensions of mesophilic lagoon bacteria along with corresponding aerobic (○) and anaerobic (●) controls.

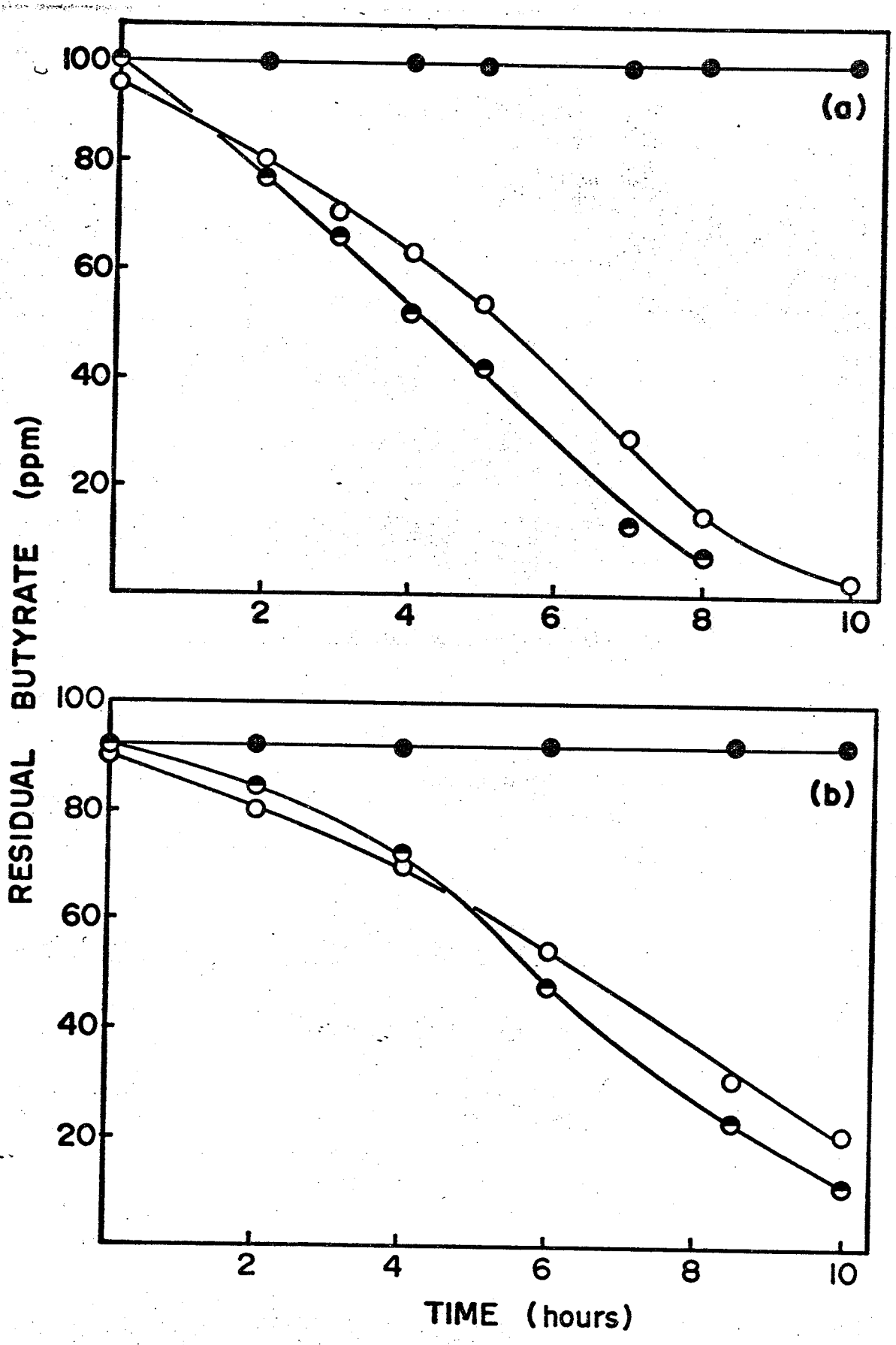
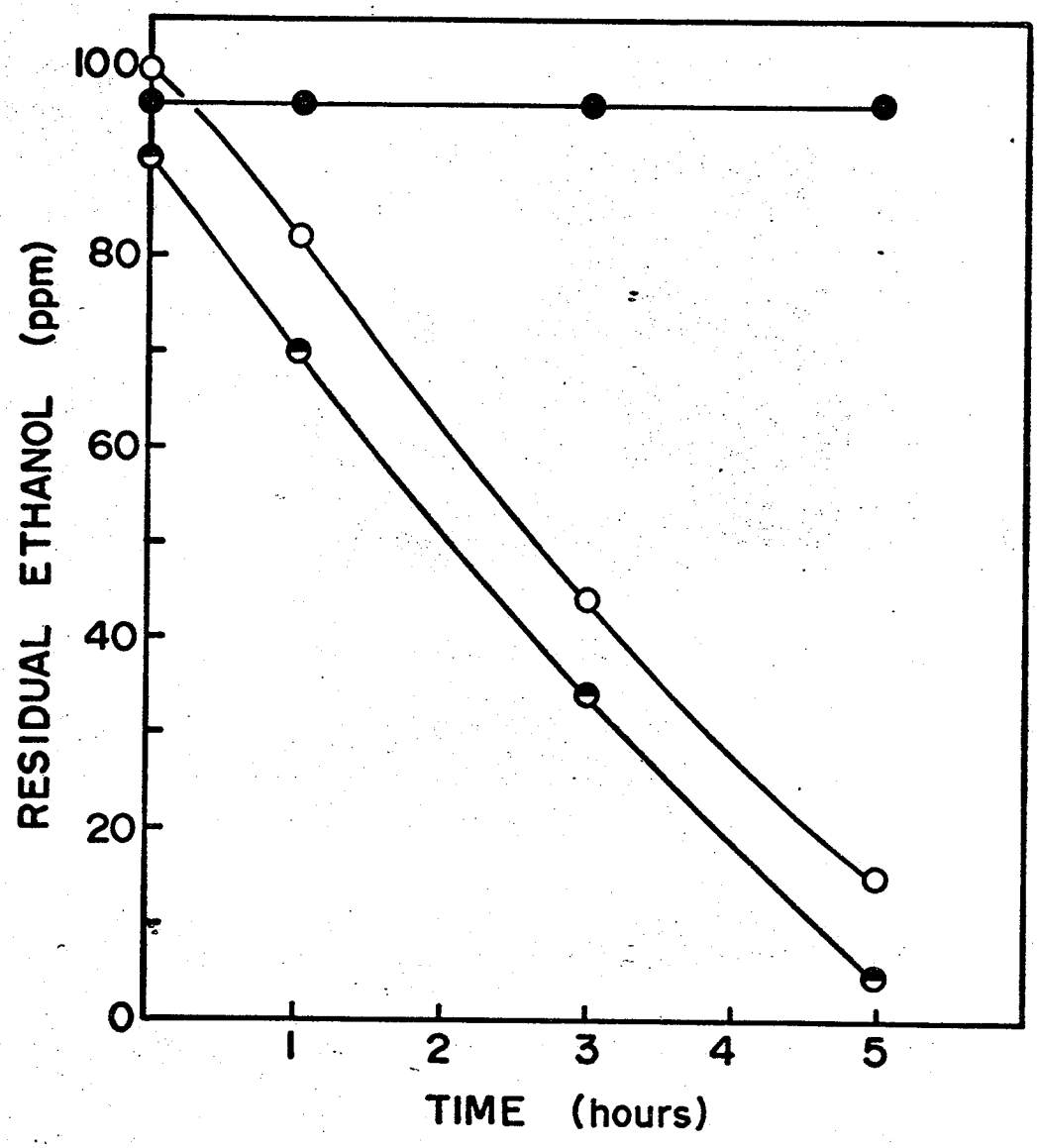

100
80
60
40
20
(a)
2
4
6
8
10
RESIDUAL BUTYRATE (ppm)
100
80
60
40
20
(b)
2
4
6
8
10
TIME (hours)

Fig. 11. Utilization of ethanol at 25°C under 0.5 ppm DO (◓) by a resting cell suspension of mesophilic lagoon bacteria along with aerobic (○) and anaerobic (●) controls.


RESIDUAL ETHANOL (ppm)
100
80
60
40
20
0
1
2
3
4
5
TIME (hours)

investigation at controlled DO levels was not carried out as no breakdown was expected.

B) Phenol

Phenol was removed aerobically but not in the absence of oxygen by psychrotrophic lagoon bacteria. The rate of degradation was very low with a lengthy lag period which ranged from 15 to approximately 40 hours depending on the cell preparation employed. The rate at 0.5 ppm DO was observed to be faster than that of the aerobic sample. This unexpected result was confirmed in a second trial at 0.5 ppm DO. The degradation rate curves for phenol aerobically, anaerobically and at 0.5 ppm DO are given in Fig. 12.

C) Benzoate

Psychrotrophic lagoon bacteria utilized benzoate under aerobic conditions but not anaerobically. A level of 0.5 ppm DO gave a rate of benzoate oxidation which was the same as the fully aerobic rate. Fig. 13 shows benzoate removal aerobically, anaerobically and under 0.5 ppm DO.

Fig. 12. Utilization of phenol at 2°C under 0.5 ppm DO (◓) by a resting cell suspension of psychrotrophic lagoon bacteria along with aerobic (O) and anaerobic (●) controls.

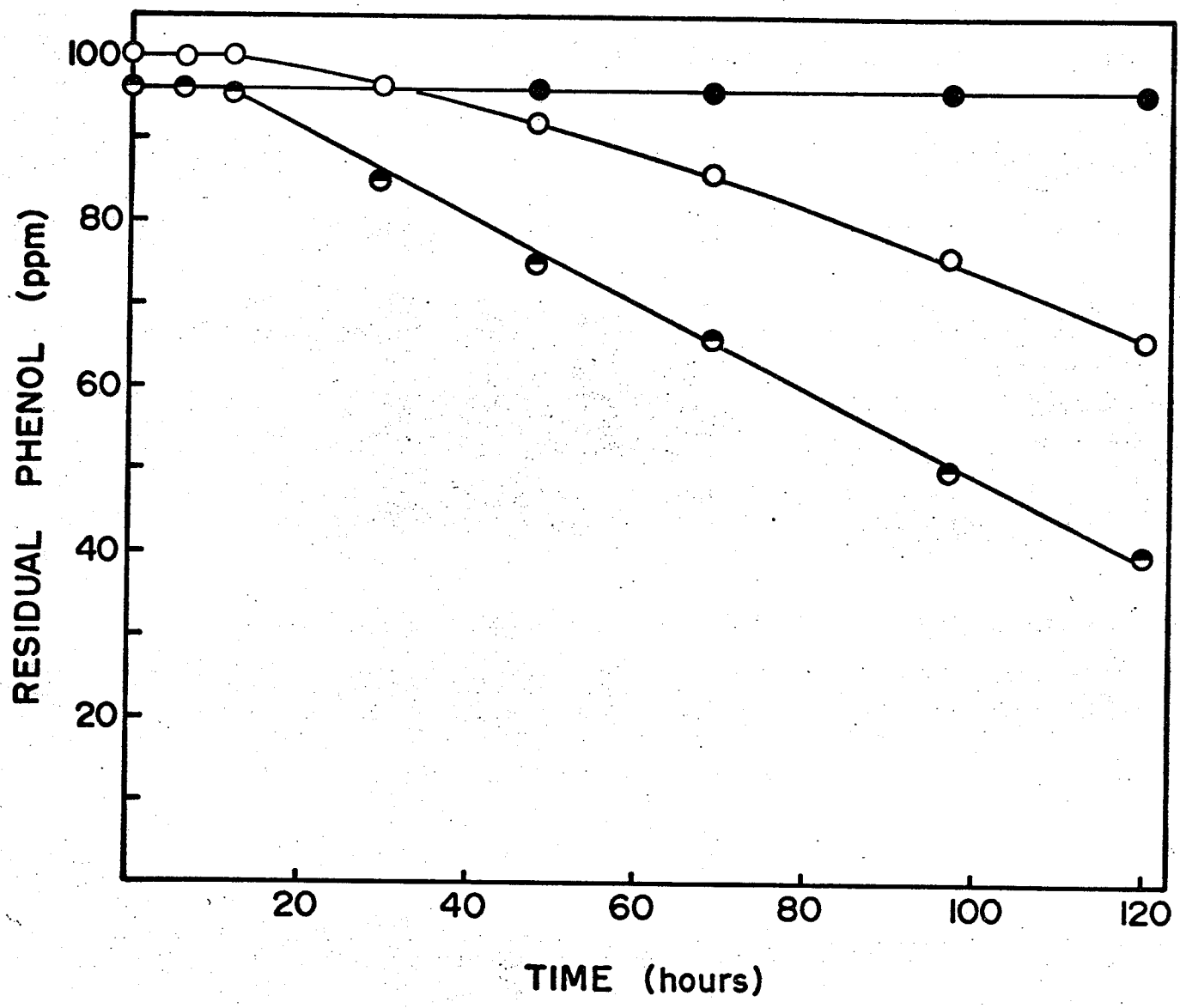
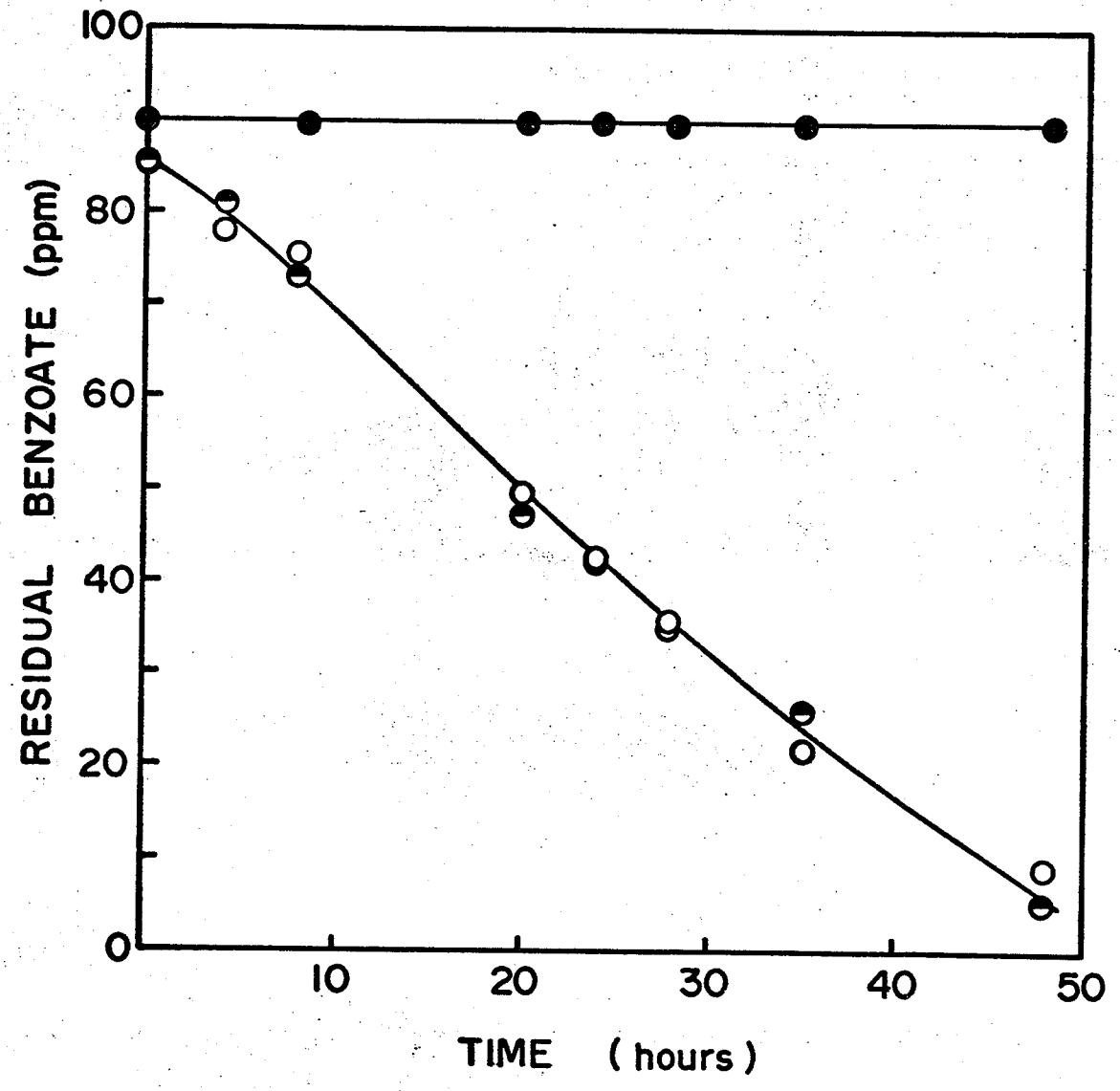

RESIDUAL PHENOL (ppm)
100
80
60
40
20
20
40
60
80
100
120
TIME (hours)

Fig. 13. Utilization of benzoate at 2°C under 0.5 ppm DO by a resting cell suspension of psychrotrophic lagoon bacteria along with aerobic (O) and anaerobic (●) controls.


100
80
60
40
20
0
RESIDUAL BENZOATE (ppm)
10
20
30
40
50
TIME (hours)

D) Acetate

Acetate was oxidized aerobically but not in the absence of oxygen by psychrotrophs. The rate of utilization was not affected by a DO concentration of 0.5 ppm. Fig. 14 shows acetate breakdown under aerobic, anaerobic and 0.5 ppm DO conditions.

E) Propionate

Propionate was degraded under aerobic conditions by psychrotrophic sewage lagoon bacteria. No utilization occurred anaerobically. At a controlled DO of 1.5 ppm the rate of propionate oxidation was analagous to that of the fully aerobic sample. Increasing the DO to 2.0 ppm did not increase the rate of breakdown over that of the aerobic control. Reducing the oxygen level to 1.0 ppm DO resulted in a rate 62 per cent that of the aerobic trial. Figs. 15a, 15b and 15c display propionate oxidation under aerobic, anaerobic, 2.0 ppm, 1.5 ppm and 1.0 ppm DO levels.

F) Butyrate

Psychrotrophic lagoon bacteria utilized butyrate aerobically. Butyrate was not metabolized under anaerobic conditions. Butyrate degradation was not

Fig. 14. Utilization of acetate at 2°C under 0.5 ppm DO (◓) by a resting cell suspension of psychrotrophic lagoon bacteria along with aerobic (O) and anaerobic (●) controls.

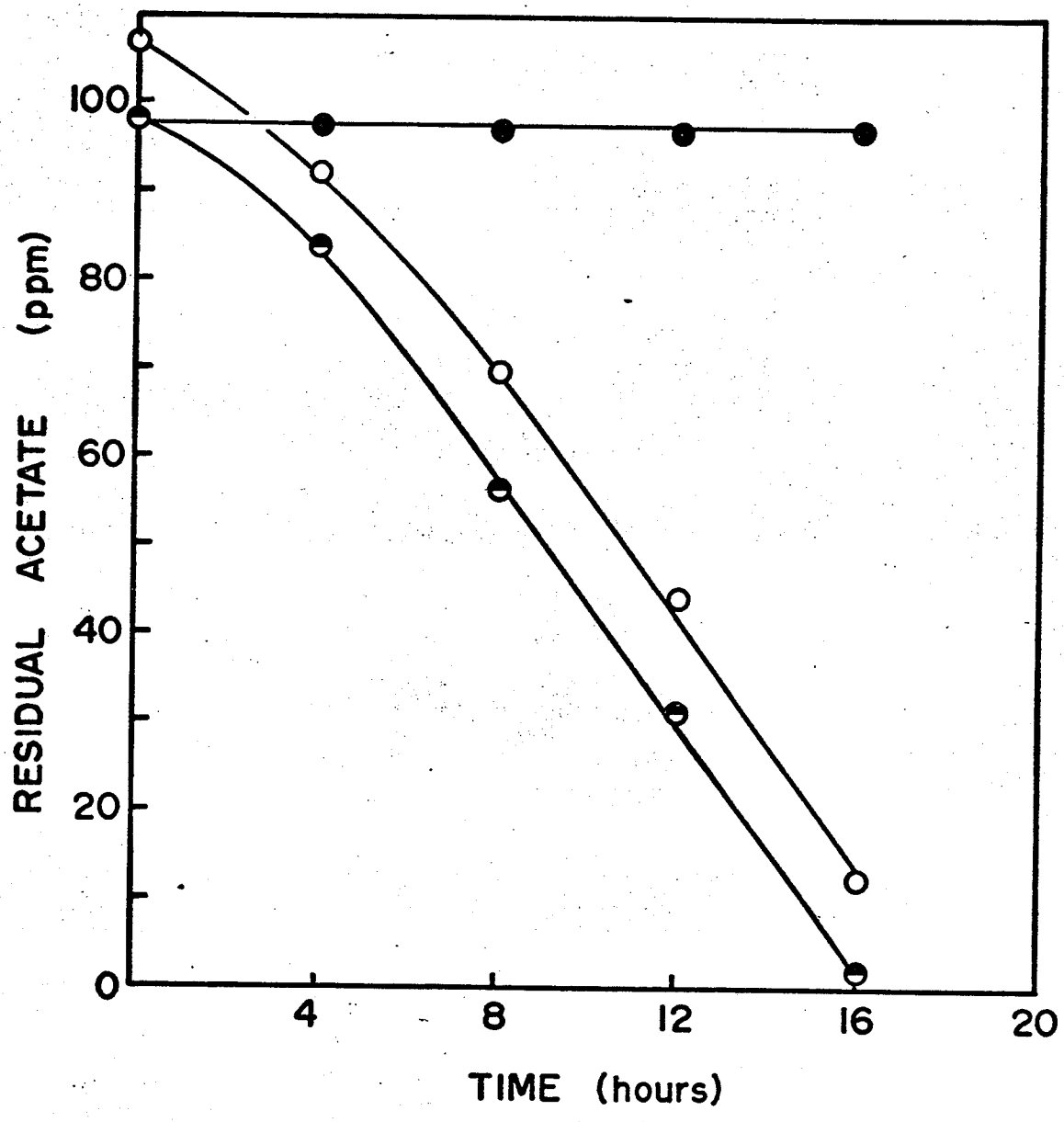
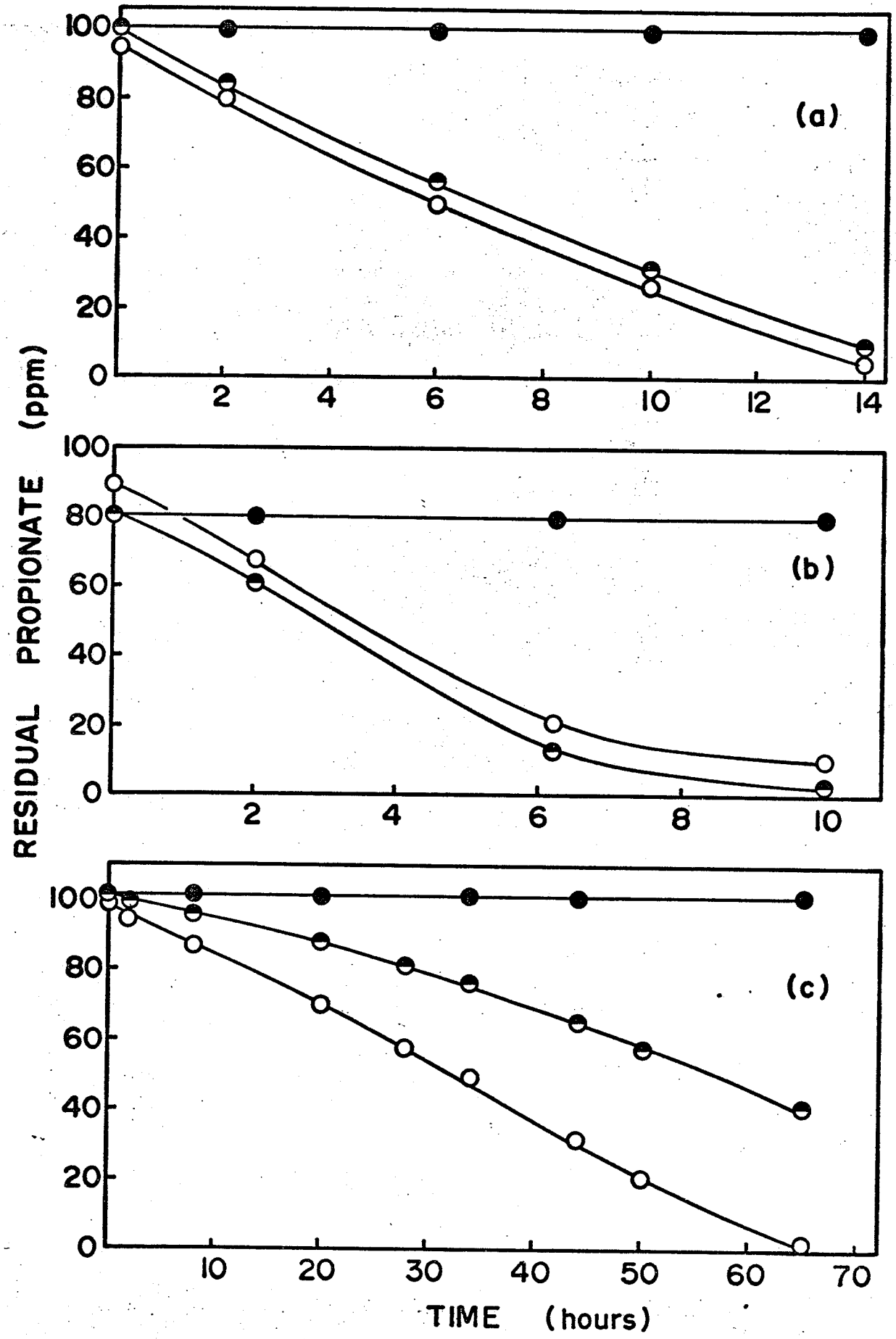

RESIDUAL ACETATE (ppm)
100
80
60
40
20
0
4
8
12
16
20
TIME (hours)

Fig. 15. Utilization of propionate at 2°C under a) 2.0 ppm DO (◓), b) 1.5 ppm DO (◓) and c) 1.0 ppm DO (◓) by resting cell suspensions of psychrotrophic lagoon bacteria along with corresponding aerobic (○) and anaerobic (●) controls.


RESIDUAL PROPIONATE (ppm)
100
80
60
40
20
0
(a)
2
4
6
8
10
12
14
(b)
(c)
TIME (hours)
10
20
30
40
50
60
70

affected by a DO concentration of 0.5 ppm. Butyrate disappearance under aerobic, anaerobic and 0.5 ppm DO levels is shown in Fig. 16.

G) Ethanol

It was found that ethanol was utilized under aerobic conditions by psychrotrophic sewage lagoon bacteria. Ethanol was not metabolized anaerobically. Degradation was seen to proceed equally well at 0.5 ppm DO as under fully aerobic conditions. Fig. 17 shows ethanol utilization aerobically, anaerobically and at 0.5 ppm DO.

IV. Diauxic Effect in Mixed Culture

It was found that when cells were challenged with a mixture of substrates, acetate, propionate, butyrate, ethanol and benzoate were utilized concurrently by a resting cell suspension of psychrotrophic lagoon bacteria at $2^{o}C$ under fully aerobic conditions. It was noted that the rate of breakdown was decreased for all substrates as compared to the rate using single substrates, with the exception of phenol and benzoate.

Phenol was degraded at a very slow rate with an approximate 15 to 20 hr lag period when used singly but did not appear to be utilized when other compounds

Fig. 16. Utilization of butyrate at 2°C under 0.5 ppm DO (◓) by a resting cell suspension of psychrotrophic lagoon bacteria along with aerobic (O) and anaerobic (●) controls.

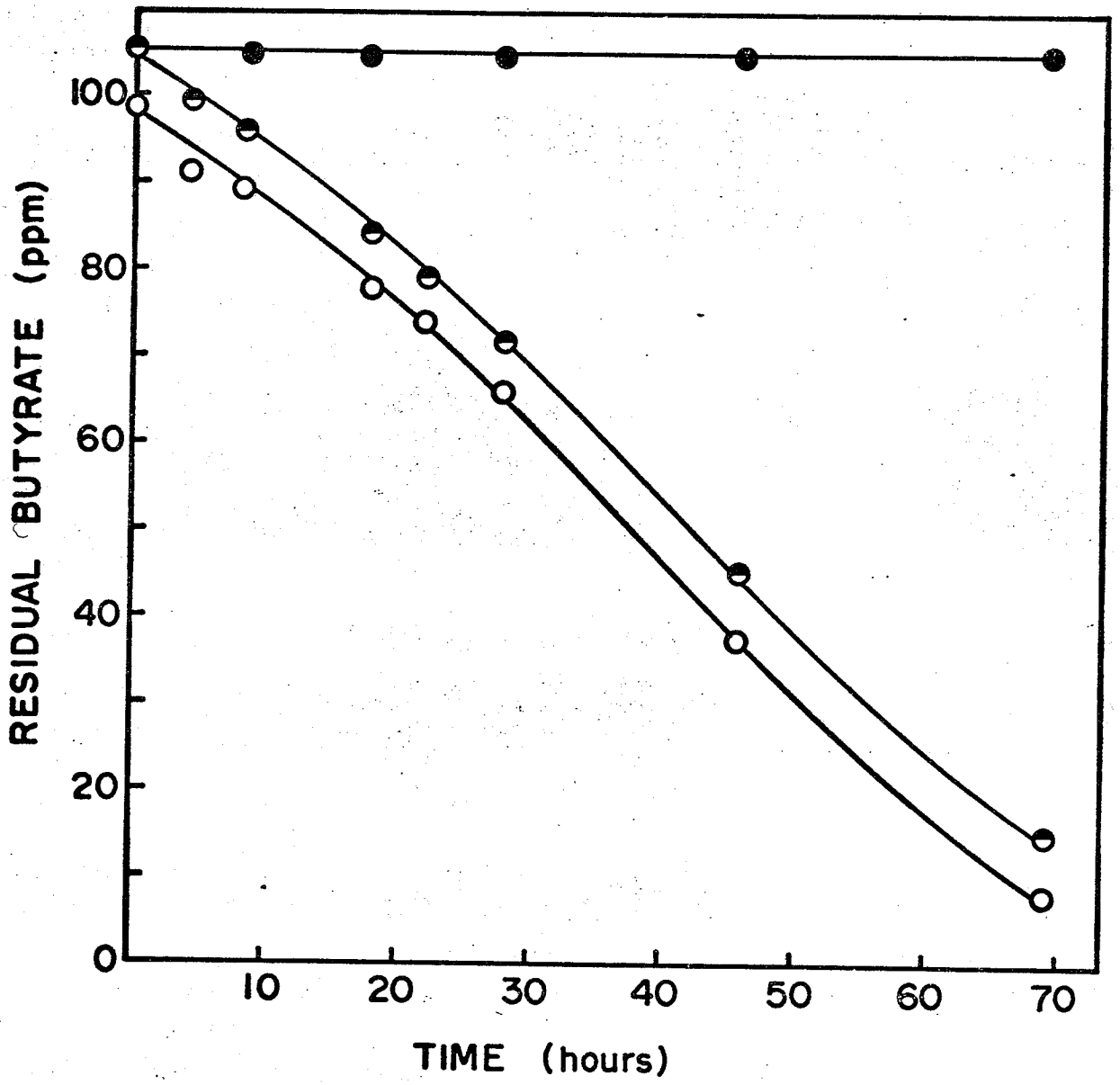
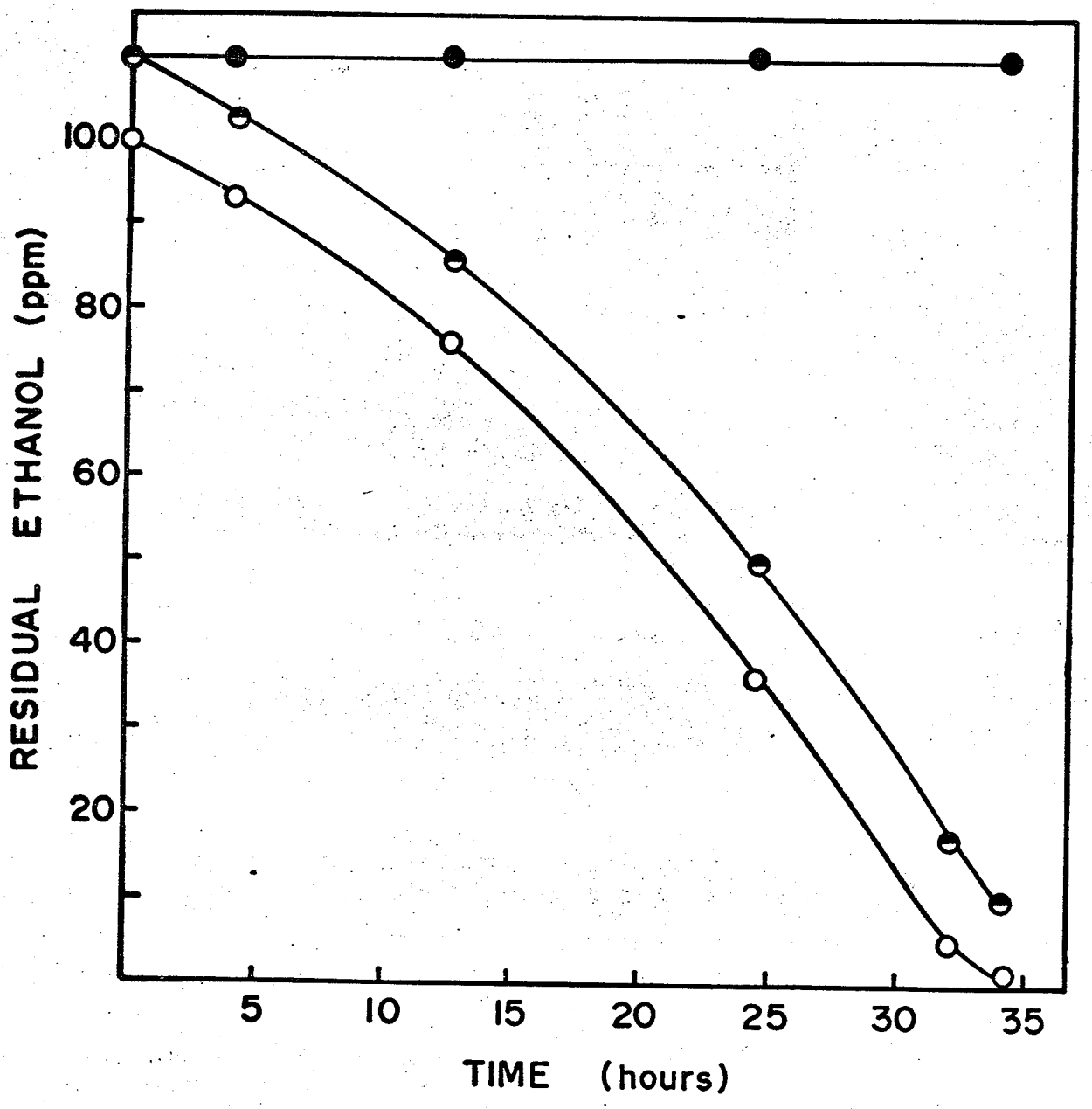

RESIDUAL BUTYRATE (ppm)
0
20
40
60
80
100
10
20
30
40
50
60
70
TIME (hours)

Fig. 17. Utilization of ethanol at 2°C under 0.5 ppm DO (◓) by a resting cell suspension of psychrotrophic lagoon bacteria along with aerobic (O) and anaerobic (●) controls.


RESIDUAL ETHANOL (ppm)
100
80
60
40
20
5
10
15
20
25
30
35
TIME (hours)

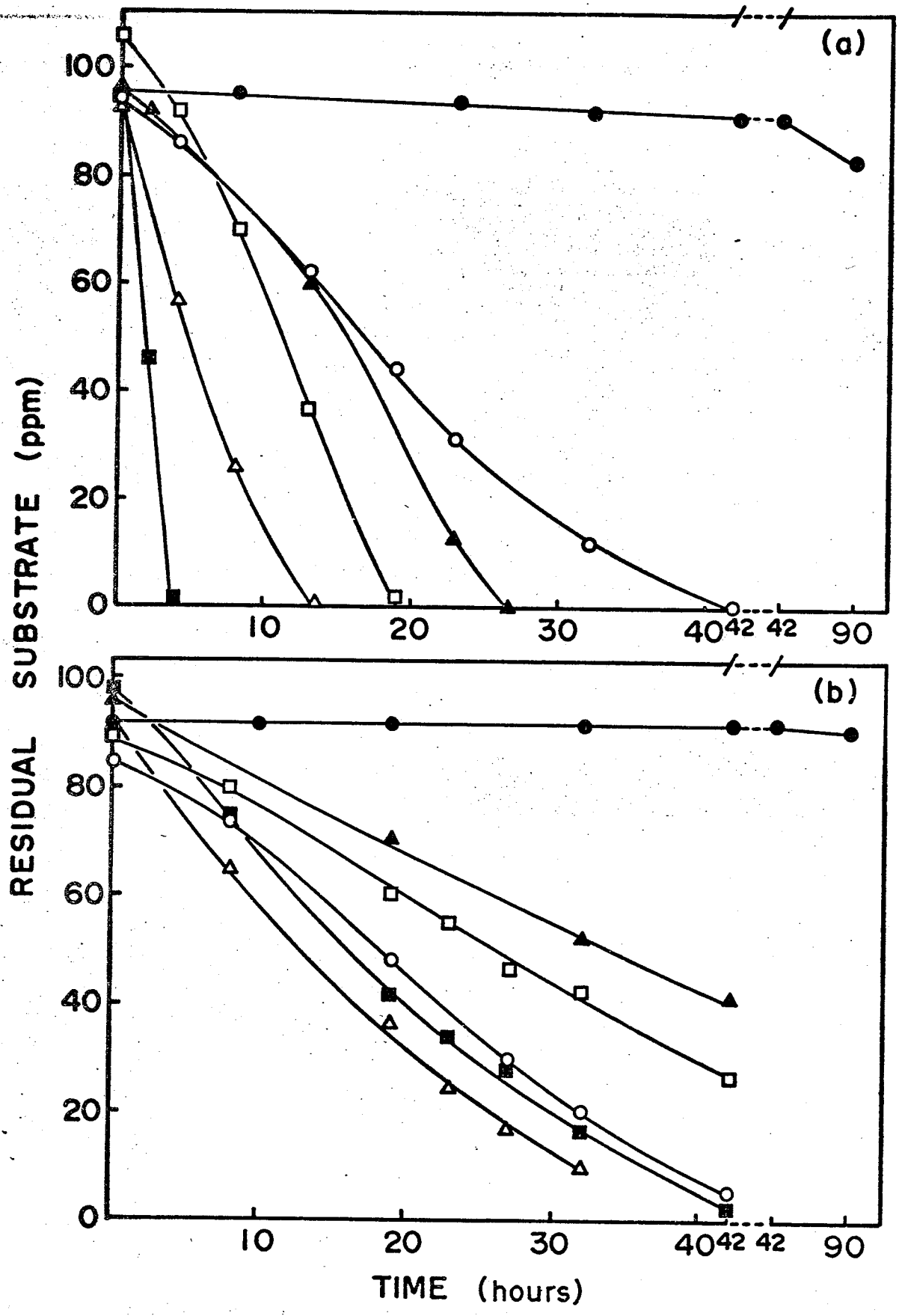
were present. Benzoate metabolism proceeded at approximately equal rates both in combination with other substrates and when used singly.

The time-course degradation plot for the differential rates of single substrates utilization is shown in Fig. 18a. The rate of breakdown plot for simultaneous mixed substrate metabolism is given in Fig. 18b.

V. Assessment of Manometric Standardization of Active Biomass

It was found that a definite correlation existed between the amount of oxygen consumed by a resting cell suspension metabolizing vitamin-free casamino acids and its demonstrated rate of substrate removal under aerobic conditions. Each substrate was associated with a specific and fairly constant rate of breakdown when a biomass correction was made. Table II gives values for substrate removal rates (col. A) and manometric oxygen uptake determinations (col. B) for resting cell suspensions of mesophilic sewage lagoon bacteria prepared at different times. Table III provides the same information for psychrotrophic resting cell preparations. These data were used to generate a constant

Fig. 18. Utilization of acetate (■), propionate (□), butyrate (Δ), ethanol (▲), benzoate (O) and phenol (●) at 2°C under aerobic conditions a) singly and b) in combination by a resting cell suspension of psychrotrophic lagoon bacteria.


(a)
(b)
RESIDUAL SUBSTRATE (ppm)
TIME (hours)
0
20
40
60
80
100
10
20
30
40
42
42
90

(col. A/col. B) for the rate of substrate removal per standardized unit of bacterial activity. The rate constants given are the maximum rates of substrate breakdown that could be expected "in situ" in the lagoon system during the summer and winter months due to the optimum conditions employed, with respect to temperature and pH, during the laboratory determinations.

Table II. Rates of substrate removal by mesophilic lagoon bacteria corrected for fluctuations in biomass.

Substrate	Trial	A Substrate removal rate at 25°C (mg hr^{-1})	B Manometric exogenous oxygen uptake at 30°C (µl O_2 hr^{-1} ml^{-1})*	A/B Rate constant (µg hr^{-1}/A_{ca})**	Average rate constant ± standard deviation
ACETATE	1[a]	6.67	35	191	
	2[b]	5.32	26	204	
	3[c]	11.63	45	258	221 ± 29.8
	4	31.25	135	231	
PROPIONATE	1[d]	25.00	135	185	
	2[e]	4.72	31	152	
	3[f]	10.50	80	132	156 ± 26.8
BUTYRATE	1[g]	12.11	80	151	
	2[h]	8.33	60	138	
	3	19.23	135	142	144 ± 6.7
ETHANOL	1[i]	19.20	135	142	142 (one trial)

Table II Continued

Substrate	Trial	A Substrate removal rate at 25°C (mg hr^{-1})	B Manometric exogenous oxygen uptake at 30°C (µl O_2 hr^{-1} ml^{-1})*	A/B Rate constant (µg hr^{-1}/A_{ca})**	Averate rate constant ± standard deviation
PHENOL	1[j]	1.40	42	33	33 (one trial)
LAS	1[k]	1.30	155	8	8.5 ± 0.5
	2[l]	1.00	108	9	

* Oxygen uptake for 1.0 ml of a 1000 x concentration cell suspension of sewage lagoon bacteria metabolizing 0.8 ml of 0.75% vitamin-free casamino acids solution.

** A_{ca} is defined as an exogenous oxygen uptake of 1.0 µl O_2 hr^{-1} ml^{-1} of cell suspension.

a-l Time-course plots of substrate utilization have been presented graphically in previous figures as follows: a, Fig. 8a; b, Fig. 8b; c, Fig. 8c; d, Fig. 9a; e, Fig. 9b; f, Fig. 9c; g, Fig. 10a; h, Fig. 10b; i, Fig. 11; j, Fig. 7; k, Fig. 6a; l, Fig. 6b.

Table III. Rates of substrate removal by psychrotrophic lagoon bacteria corrected for fluctuations in biomass.

Substrate	Trial	A Substrate removal rate at 2°C (mg hr^{-1})	B Manometric exogenous oxygen uptake at 15°C (µl O_2 hr^{-1} ml^{-1})*	A/B Rate constant (µg hr^{-1}/A_{ca})**	Average rate constant ± standard deviation
ACETATE	1	2.56	50	51	
	2	17.00	172	99	72 ± 20.3
	3	11.10	114	97	
	4	2.43	46	53	
	5[a]	6.58	108	61	
PROPIONATE	1	1.96	50	39	
	2	6.25	172	36	
	3	5.0	114	43	41 ± 2.7
	4	1.90	46	41	
	5[b]	6.67	150	44	
	6[c]***	8.70	50	174	
	7[d]	1.65	40	42	
BUTYRATE	1[e]	1.43	50	29	
	2	8.33	172	48	38 ± 11.0
	3	5.26	114	46	
	4	1.25	46	27	

Table III. Continued

Substrate	Trial	A Substrate removal rate at 2°C (mg hr^{-1})	B Manometric exogenous oxygen uptake at 15°C (µl O_2 hr^{-1} ml^{-1})*	A/B Rate constant (µg hr^{-1}/A_{Ca})**	Average rate constant ± standard deviation
ETHANOL	1	0.91	50	18	
	2	4.00	172	23	
	3	2.78	114	24	23 ± 3.1
	4	1.16	46	25	
	5[f]	2.90	108	26	
BENZOATE	1	0.69	50	14	
	2	2.94	172	17	16 ± 1.7
	3	2.00	114	18	
	4[g]	1.92	118	16	
PHENOL	1	0.05	50	1.0	
	2	0.26	172	1.5	
	3	0.18	114	1.6	1.45 ± 0.31
	4[h]	0.36	215	1.7	

Table III. Continued

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* Oxygen uptake for 1.0 ml of a 1000 x concentration cell suspension of sewage lagoon bacteria metabolizing 0.8 ml of a 0.75% vitamin-free casamino acids solution.

** A_{ca} is defined as an exogenous oxygen uptake of 1.0 µl O_2 hr^{-1} ml^{-1} of cell suspension.

*** Data from this trial were not used in calculating the average rate constant (see pg. 98).

a-h Time-course plots for substrate utilization have been presented graphically in previous figures as follows: a, Fig. 14; b, Fig. 15a; c, Fig. 15b; d, Fig. 15c; e, Fig. 16; f, Fig. 17; g, Fig. 13; h, Fig. 12.

DISCUSSION

DISCUSSION

The main objective of this investigation was to determine the effect of dissolved oxygen concentration on the rate of breakdown of selected organic compounds by bacteria indigenous to a sewage lagoon. The catabolism of all of the substrates selected for study is mediated by molecular oxygen. Benzoate and phenol were chosen as representative aromatic moieties that might be residues from the stabilization of synthetic chemicals or naturally occurring organics present in raw sewage. Acetate, propionate, butyrate and ethanol represent compounds which would accumulate in the lagoon under anaerobic conditions. LAS was chosen because this commercial detergent represents the major portion of MBAS being loaded into the lagoon. The design of the experimental system allowed for the examination of catabolic activity as a function of oxygen concentration. It was assumed that all other parameters remained constant and that the stirring or shaking of suspensions was sufficient to keep the cells in a dispersed state so that diffusion of oxygen to cell surfaces would not become a limiting factor.

In the majority of experiments carried out, trials with propionate being the exception, actual critical DO levels were not realized. That is to say, in most cases, the lowest DO concentration that was imposed upon the bacterial cells did not significantly alter the rate of utilization of the test organic compounds when compared to the rate of utilization under fully aerobic conditions.

Trials with summer mesophilic bacteria at 25°C revealed the critical DO concentrations to lie in the region $\leqslant$ 0.24 ppm for the oxidation of LAS detergent, phenol and acetate. Trials with ethanol and butyrate showed an oxygen requirement of $\leqslant$ 0.5 ppm. Values for ethanol and butyrate may have been as low as 0.24 ppm but unfortunately samples taken for these substrates at 0.24 ppm DO were lost due to the breakdown of freezing facilities used for storage. Samples containing benzoate met the same fate and as a result no data on benzoate metabolism by summer bacteria are available. Mesophilic activity with propionate as the substrate disclosed a critical value of $\simeq$ 2 ppm DO. As can be seen (Fig. 9a) the rate of propionate oxidation under 2.0 ppm DO appeared to be slightly lower than that of the fully aerobic control

but this small difference was regarded as being insignificant. A progressively decreasing rate of propionate breakdown was observed as the DO was lowered from 2.0 ppm to 1.5 ppm (Fig. 9b) and then to 1.0 ppm (Fig. 9c) clearly showing the retarding effect of oxygen concentration below the critical level on reaction rate.

Experiments with winter pychrotrophic bacterial suspensions at 2°C showed that the biological oxygen requirement for the degradation of acetate, butyrate, phenol, ethanol and benzoate was met at a level of 0.5 ppm, the lowest DO concentration studied. It was decided that further investigation below this concentration would be of little practical importance with respect to actual aerated lagoon operation under winter conditions. Critical values may indeed have been located well below this level as one might suspect that at low temperatures the reduction of respiration rate might result in a decreased need for molecular oxygen. Kempner (48) working with Micrococcus candicans and Winzler (93) using washed yeast suspensions have shown that the critical oxygen tension for respiration in these microorganisms was lowered by a decrease in temperature. Experiments with winter bacteria utilizing

propionate at 2°C indicated the critical DO level to be > 1.0 ≤ 1.5 ppm. This value, being lower than the needs of summer bacteria for propionate oxidation at 25°C, lends support to a decreased requirement for DO at low temperatures but further data for other substrates would be needed as conclusive evidence.

None of the substrates tested were degraded anaerobically as would be expected considering the known oxygen dependent mechanisms of aromatic ring cleavage (3, 9, 25, 66, 67, 85), and β-oxidation of straight chain aliphatic compounds (33, 47, 69) giving rise to the oxidative regeneration of reduced pyridine nucleotides and formation of precursors for the TCA cycle. Acetate and compounds such as ethanol that can yield Acetyl-CoA can enter the cycle directly and then be completely oxidized (1, 74).

Constitutive enzymes which are present in appreciable quantities at all times in bacterial cells are responsible for the catabolism of acetate, propionate, butyrate and ethanol. Aromatic metabolism on the other hand is dependent on induced enzyme formation, i.e. prior conditioning to the substrate or some related compound, before breakdown occurs (27, 28, 45, 67). The results in this study were in good agreement with the above

mechanisms. Utilization of aliphatic substrates in all instances was observed to begin almost immediately. Phenol exhibited an induction period at 25°C (Fig. 7) and a longer period at 2°C (Fig. 12) during which no breakdown occurred. There did not appear to be any lag period associated with benzoate metabolism (Fig. 13) suggesting that the respective enzymes were synthesized during growth of the bacteria "in situ" in the lagoon.

Unexpected observations were noted in the case of butyrate metabolism by mesophiles (Figs. 10a and 10b) and phenol utilization by psychrotrophs (Fig. 12). In both instances the rate of breakdown was observed to be faster at 0.5 ppm DO than under fully aerobic conditions. The same phenomenon occurred for butyrate at 1.0 ppm DO. This response could not have been an experimental artifact since the same result was obtained in duplicate trials for both compounds. These results might be explained by the proposals of Harrison et al (39, 43) who inferred that respiration and subsequently catabolism may increase at DO concentrations below a critical level in order to provide enough energy to meet cellular requirements. Another possibility which would be more applicable for phenol is that the combination of low oxygen concentration, low temperature and high phenol concentration

place the organism under some form of stress. A high respiration rate might then serve to more rapidly reduce this inhibitory substrate to less toxic levels. That no increase in butyrate catabolism at 0.5 ppm DO by winter bacteria was noticed could indicate that the critical DO level for this substrate is lower for psychrotrophic bacteria than for mesophiles.

During the early 1960's linear alkylbenzene sulfonates (LAS) gradually replaced their microbial resistant branched chain ABS predecessors as a major surfactant in laundry detergent formulations (84). Since that time an appreciable amount of research has been done supporting the environmental acceptability of LAS based products (32, 84).

The findings in the present study that LAS was readily metabolized by summer mesophilic bacteria at 25°C under aerobic conditions and not used at all by resting cell suspensions of psychrotrophic sewage lagoon bacteria at 2°C are in agreement with the observations of other workers using both resting cells (37) and growth conditions (56). From the available data it would seem that the ability of mesophilic organisms to degrade LAS is at least partly dependent on temperature and independent of DO concentration down to very low levels. The oxygen dependency of

LAS breakdown is amply demonstrated by its failure to be utilized anaerobically. Resting cell suspensions of psychrotrophic sewage lagoon bacteria might appear unable to degrade LAS. It has recently been shown, however, that under growth conditions in a small-scale activated sludge unit (83) LAS type compounds can be substantially degraded at temperatures as low as 5°C after a period of acclimatization. The use of an experimental system employing resting cell conditions for the study of LAS breakdown at low temperatures presents a limitation in that the bacterial cells remain active for comparatively short periods of time. The use of a growing cell system would be more advantageous. Cell viability would not then become a critical factor in experiments of this type where the bacteria require a period of time for acclimatization before degradation can be initiated.

It has been reported (16) that growing mixed cultures of microorganisms isolated from a polluted stream preferentially utilized glucose to other carbon sources such as lactose and butyrate. Investigations into diauxic effect in mixed cultures of lagoon bacteria revealed that when a resting cell suspension was challenged with a multiple substrate condition the

general rate of catabolism was reduced compared to the rate using single substrates (Figs. 18a and 18b). Most compounds were degraded concurrently, however, with the exception of phenol which was not utilized suggesting that other energy sources were used preferentially over phenol. The reduction in removal rate of other substrates might be due to a toxic effect exerted on the bacterial cells by a high phenol concentration. Phenol concentrations in excess of 20 mg/l have been shown to produce a reduction in the activity of activated sludge organisms (26). Benzoate utilization was not significantly affected attesting to the fact that, although individual species carry out specialized roles, the overall physiological potential of microorganisms involved in waste stabilization is vast. LAS detergent was not included as a component in the mixed substrate trial because it was previously determined that no breakdown occurred at $2^{o}C$.

The manometric standardization procedure, where exogeneous oxygen uptake was the criterion for activity, provided a reasonable correction for fluctuations in biomass. The method appears to fulfill the demands of a workable bioassay in that the variable measured (O_2 uptake) 1) was dependent on metabolic activity and viable biomass and 2) reflected the rate of substrate removal. Dead cell material, dissolved organics and

nonbiological particulates were corrected for by substracting the endogenous respiration from the total oxygen uptake obtained with casamino acids substrate. As expected a different (distinct) rate of breakdown was associated with each substrate. When corrected for fluctuations in biomass, the rate was relatively constant for each compound as witnessed by the rate constant values in Tables II and III. Although it appears from the data that the rate of summer (25°C) substrate utilization is higher than that during the winter (2°C) no valid comparison can be drawn because of the different conditions employed for the respective biomass estimations. It is evident, however, that substantial rates of breakdown can occur at low temperatures. Variations in results shown in Tables II and III might be attributed to particulate materials recovered from the lagoon water along with the bacteria. Data for Trial 6 in Table III were not included in calculating the average rate/activity constant because this suspension displayed a rate of propionate utilization considerably greater than would have been expected on the basis of its exogenous oxygen uptake. Possibly this preparation contained higher proportions of propionate metabolizers than is normally encountered.

A salient feature of a mixed bacterial culture is that each member of the mixture has its own specific physiological spectrum, i.e. can metabolize a limited number of compounds. A wide variety of substrates can be utilized in mixed culture but only a portion of the total species present will be able to use any one substrate. In the manometric procedure a multiple substrate would then best serve to demonstrate total biomass. Amino acids are assimilated by many types of bacteria (35, 44, 72). Therefore, for oxygen uptake determinations, vitamin-free casamino acids was chosen as a convenient source of a multiple substrate. Greater numbers of organisms would then be expected to metabolize some component of the mixture than had a single substrate been used.

In evaluating the data presented in this report it must be appreciated that the organisms used were not in a growing state. The oxygen demands and rates of biochemical reaction may be quite different in the actual lagoon situation where a large number of parameters are available to exert their influences on growing cells. The parameters which normally regulate bacterial growth in the lagoon (i.e. pH, temperature and substrate availability) were controlled in the present study to approximate optimum conditions. "In situ" in the lagoon,

changes in pH and lowered temperatures can only result in a reduction in the growth rate and activity of indigenous microorganisms. This would lead to a requirement for dissolved oxygen less than that indicated in this study because some factor other than DO concentration has become the rate-limiting parameter. This does not indicate that the findings cannot be used, but rather that they do not provide an absolute index with regard to lagoon management. However, it would in all probability be safe to assume that the relationships demonstrated in this study could be extrapolated within reasonable limits to the "in situ" system. Proceeding from this assumption, an evaluation of the aeration requirements for the Charleswood Lagoon operation can be made.

Based on the data obtained it would appear that during the summer months maintaining a level of 2.0 ppm DO in the aerated system is justified. Efficient metabolism of propionate by summer resting cell suspensions required a DO concentration of approximately 2 ppm while for other substrates the requirement was considerably less.

Under very cold conditions as experienced in the lagoon during the winter months the aeration system is probably supplying oxygen in excess of biological process

demands. The combination of reduced respiration rates of psychrotrophic bacteria (36) and the greatly increased solubility of oxygen at low temperatures (81) lends support to the above statement. In this study a level of 1.5 ppm DO was sufficient to achieve efficient degradation of propionate by psychrotrophic lagoon bacteria. The rate of metabolism of other substrates was not affected by a DO concentration of 0.5 ppm. Where an air-water interface exists as in the aerated lagoons it is unlikely that DO concentrations could fall to levels low enough to retard BOD removal. The main function of aeration supplied by the Air Aqua, Air-Gun and Surface Aerator installations during winter operation appears to be related more to the mechanical function of mixing and maintenance of ice-free conditions in lagoon cells rather than meeting biological requirements.

It should be noted that a level of 0.5 ppm DO did not reduce the rate of breakdown of any of the substrates tested, propionate being the exception, at either 25^{o}C or 2^{o}C. Trials involving propionate were not carried out at levels as low as 0.5 ppm DO. But from the pattern of decreasing breakdown rate with decreasing DO concentration exhibited in the text it

would seem that propionate could still be degraded at 0.5 ppm DO although at a reduced rate. A DO concentration of 0.5 ppm is only 25 per cent of the minimum operating level in the Charleswood Lagoon. It is therefore possible that reduced rates of aeration could be employed without serious effect on the overall efficiency of biological stabilization. An aeration rate that introduces 0.5 ppm DO might also generate a mixing level sufficient to meet wastes engineering standards.

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